

**Factors Influencing Growth and Fruiting
of Coprinus lagopus Fr.**

A Thesis presented by

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A. INTRODUCTION.

Experimental studies upon fruiting in the Hymenomycetes have mostly been concerned with the physiology of the developing or mature sporophore. There has been some work, though little compared with that in certain other major groups of fungi, in which the nutritional requirements of vegetative mycelia of these organisms have been studied. However, little has been done to determine those factors responsible for or associated with the commencement of fruiting, or to elucidate the metabolic processes connected with the transition from vegetative to reproductive growth. These aspects of the problem of fruiting form the subject of the present investigation.

Coprinus lagopus was chosen for these researches because of its readiness to fruit under laboratory conditions, the convenient size of its fruit-bodies, and the brevity of its life-cycle.

Kleb's (1900) views on reproduction in fungi (Coons, 1916) are that it is the external conditions which mainly determine whether fruiting takes place; that conditions which favour reproduction are always more or less unfavourable for vegetative growth; that vegetative growth may take place under a wider range of environmental conditions than reproduction; and that vegetative growth appears to be mostly a preliminary for the initiation of reproduction, in that it creates a suitable internal environment for it.

How well these principles apply to C.lagopus will be considered in the light of the following investigations.

The nutritional requirements of this fungus have already been studied to some extent by other workers, but in certain respects there is a lack of unanimity in the results obtained.

Voderberg (1948) found that growth and fruiting of this organism in partially chemically defined media was dependent upon the presence of cellulose in the form of finely ground straw or blotting paper. The presence of powdered straw appeared to permit some use of simpler carbohydrates which were otherwise not usable.

Voderberg stated that nitrate-, ammonium-, and organic-nitrogen could be used, although best growth and fruiting took place with organic nitrogen sources.

On the other hand, Leonian and Lilly (1938) successfully cultured the mycelium of C.lagopus on an agar medium in which a simple sugar, glucose, was the carbon source, and a mixture of amino acids provided nitrogen. The fungus was described by these workers as being unable to use either ammonium- or nitrate-nitrogen. No fruiting was reported upon any of the media used.

The vitamin requirements of C.lagopus were also investigated by Leonian and Lilly (1938) who found that aneurin alone was necessary for growth.

Other aspects of its physiology which have been described include that it grows and fruits best at 25°C., and that the initiation of fruiting is virtually independent of light

(Voderberg, 1949). This contrasts with Coprinus nycthemerus in which fruiting does not commence in darkness (Brefeld; 1889). Light does, however, play a part in every subsequent stage of development of the individual fruit-body of C.lagopus (Voderberg, 1950). If light is withheld, the sporophores become etiolated, having long and slender stipes bearing rudimentary pilei. Temperatures above 25°C. have the same effect as light in this respect, permitting normal development (Borris, 1934 b). In its capacity both as a formative and as a tropic stimulus, only light of a wavelength longer than that of red evokes a response (Borris, 1934 b).

Concerning the initiation of fruiting in C.lagopus, Voderberg (1949) concluded that it was independent of the quantity of available food substances, since cultures on different quantities of manure gave rise to sporophore primordia simultaneously. On the other hand, a certain amount of mycelium, corresponding to that formed on at least 1.5 g. of manure, was indispensable. This result is compatible with Buller's view (1931, p. 152 et seq.) that fruiting could take place in the larger fungi only when there was sufficient vegetative mycelium to support a fruit body of a certain minimal size.

In a number of fungi, exhaustion of the substrate has been found to be conducive to the initiation of fruiting. Wakefield (1909) found that this was so for two species of Hymenomycetes grown in pure culture, viz. Schizophyllum commune and Stereum purpureum. In other Hymenomycetes fruiting took place sooner

with low concentrations of sugar in the medium than with high (Plunkett, 1953; Bille-Hansen, 1953).

This review of literature has been almost entirely confined to pertinent investigations on Hymenomycetes. Further reference will be made in the body of the text to immediately relevant work on fungi of other groups. To review comprehensively the problems of fruiting in fungi in general would occupy more space than is here appropriate.

B. MATERIALS AND GENERAL METHODS.

I. Origin and Identity of the Isolate.

The organism used throughout this work was isolated from horse dung which was incubated at laboratory temperatures with natural illumination for three weeks by which time its sporophores had appeared. Pure cultures were established each with a number of basidiospores taken from a sterile glass slide upon which they had been shed by a mature pileus

There is a good deal of confusion in the literature concerning the nomenclature of Coprinus lagopus and its allies. The difficulty in attaching a name to one of these organisms stems from their morphological similarity and also from statements concerning the substrates upon which these species are to be found.

Briefly, there are two groups of names. The one contains a single name, C.lagopus Fr., which is described in most of the literature as being a rootless form not found upon dung (Lange, 1915, 1935; Konrad and Maublanc, 1924-37; Wakefield and Dennis, 1950). The other group of names consists of C.macrorhizus (Pers.) Rea, C.fimetarius (L.) Fr., C.fimetarius Fr. v.macrorhizus Sacc., and C.cinereus (Schaeff.) Fr., all of which are used as synonyms of a rooted species which is usually found on dung. The name C.cinereus is not rejected by Pearson and Dennis (1948) as being a valid name of yet a third species, although one of these authors subsequently mentions the use of this name as a synonym by Konrad and Maublanc, and presumably inclines to the same view (Wakefield

and Dennis, 1950). It has been suggested (Lange, 1935) that this use is questionable, since Fries originally used C.cinereus for a rootless form, and Buller (1924) is inclined to believe that this name has been used for organisms which he would call C.lagopus.

In the literature on experimental work in the Hymenomycetes, the name C.lagopus has been applied to a coprophilous fungus by a number of workers (Brefeld, 1877; Buller, 1924; Borriss, 1934 a, b; Voderberg 1948, 1949, 1950), and it appears from the illustrations in a number of these works that there is little doubt that these organisms were C.lagopus as understood by Lange, Konrad and Maublanc, and Rea (1922), although only the last mentioned worker reports that the fungus he understands by this name occurs upon dung.

Cultured upon horse dung, the isolate used in this work produced sporophores of which the following is a brief description:-

Pileus usually about 1.5 cm. diameter; at first cylindric-oval, then campanulate with a somewhat pointed apex; initially white, owing to the presence of dense white down, becoming grey during expansion.

Stipe usually 10-14 cm. high; tapering gradually upwards to a diameter of about 2 mm.; white, hollow, very fragile; densely clothed with fibrillae when young, but more sparsely when mature.

Spores 13.5 - 15.5 x 7-8 μ ; black, smooth.

This description closely agrees with that given by Buller (1924) for Coprinus lagopus. There is the further agreement in respect of the capacity of this isolate of producing under certain conditions dwarf fruit-bodies, sometimes little more than 1 cm. high and slender in proportion (Buller, 1922, 1924).

The organism used in this work will therefore be referred to as Coprinus lagopus Fr.

II. Cultural Techniques.

a) The choice of media for maintenance of culture and for experimental work.

Initially the isolate was maintained on sterilized horse dung in Petri dishes. Fresh horse dung was pressed to express free liquid, and sufficient was placed in each plate to provide a compact layer about 5 mm. deep. The plates were then autoclaved for 20 minutes at 15 lb./sq.in. pressure, and each subsequently inoculated in the centre with a number of basidiospores. Several spores were used because C.lagopus is heterothallic (Mounce, 1922). On this substrate, sporophores began to develop in the light on about the 10th. day and matured by about the 14th. day.

Because of the inherent variability of this substrate, it was found necessary to devise an agar medium which would enable the conditions in experiments upon the effects of physical factors to be more readily standardized. Preliminary results indicated that growth of the organism was deleteriously affected by an initial pH less than 6.5, and completely inhibited below

pH 4.5. The following medium prepared from yeast extract had a favourable pH by virtue of the use of a suitable phosphate, and supported dense vegetative growth and good fruiting. It was therefore adopted throughout the work as the medium on which stock cultures were maintained.

0.3% Yeastrel Agar:

Glucose	1%
*"Yeastrel"	0.3%
K ₂ HPO ₄	0.2%
MgSO ₄ ·7H ₂ O	0.02%
Agar	2%

(* "Yeastrel" is prepared by the Brewers' Foods Supply Company, Ltd., Edinburgh.)

Experiments showed that concentrations of "Yeastrel" less than 0.3% led to thinner mycelial growth, while higher concentrations gave no appreciable betterment of vegetative or reproductive growth.

An analysis of "Yeastrel" kindly supplied by the manufacturers showed that this product contained considerable quantities of amino acids. A number of experiments revealed that dl- α -alanine alone could function as the nitrogen source in the medium in place of "Yeastrel", provided that an external source of aneurin was available. Accordingly, the constitution of the medium described above was modified for use in experimental work, the "Yeastrel" being replaced by 0.1% dl- α -alanine and 50 μ per 100 ml. of medium of aneurin hydrochloride. This medium is subsequently referred to as "0.1% Alanine Agar", or if the agar is omitted, "0.1% Alanine Liquid Medium".

Wherever possible, chemicals of analytical reagent quality were used, although no differences were detected between growth in 0.1% Alanine Liquid Medium prepared from ordinary laboratory chemicals, and in that prepared from analytical reagents.

b). Culture vessels.

For experiments in which no manipulation of the colony was entailed, 10 oz. medical flats (approximately 13 x 6 x 3.5 cm.) laid upon their sides were used. Each contained 20 ml. of medium, whether liquid or solidified with agar. The necks of the medical flats were plugged with cotton wool.

In the case of those experiments in which it was necessary to manipulate the colony, Petri dish cultures were used, again with 20 ml. of medium per dish.

c) Methods of sterilization.

Since the medium was deliberately made slightly alkaline by the use of an appropriate phosphate salt, the caramelization of the glucose which would occur on autoclaving and which might influence the growth of the fungus was obviated or minimized throughout this work by sterilizing either by steaming for half an hour on three successive days, or by keeping the glucose and phosphate in separate fractions of the medium during autoclaving at 15 lb./sq.in. for 20 minutes, and combining the fractions aseptically afterwards. Lewis (1930) showed that such methods prevented the formation of substances inhibitory to a species of bacterium which were otherwise formed when glucose and K_2HPO_4

were autoclaved together.

C.lagopus can utilize the thiazole and pyrimidine moieties of aneurin which are formed when aneurin undergoes alkaline hydrolysis (Leonian and Lilly, 1938), and consequently whether aneurin was added to the rest of the medium before sterilizing or afterwards was immaterial. In the majority of cases it was added to the medium prior to sterilization.

d) Methods of inoculation.

Apart from preliminary work upon sterilized dung when inocula composed of a number of spores were used, mycelial inocula were used throughout this work. Small plugs of agar bearing the vegetative mycelium of the fungus were taken at a uniform distance of 3 mm. from the margin of a plate culture on 0.3% Yeastrel Agar. The plugs were cut out with a glass tube of 1 mm. internal diameter. A single plug was placed in each culture vessel. In agar cultures, this was placed centrally upon the medium unless stated otherwise in the text.

The age of the cultures at the time of removing the 1 mm. plugs for inoculation was not standardized, but was usually between one and two weeks.

e) Incubation.

Except where stated otherwise, cultures were maintained at 25°C., with continuous illumination at about 8 foot-candles provided by an incandescent filament bulb.

III. Methods of Assessing Growth and Fruiting.

In most experiments, radial growth of the fungal colonies

was recorded at intervals, and where of especial interest, the results are presented with the other data. Each radius was determined from the mean of two diameters intersecting at 90° in plate cultures, or along the diagonals in the case of rectangular medical flats.

It was soon found that measurements of radial growth alone often gave a misleading index of the vigour of growth upon various media or under different conditions. Wherever possible therefore additional data on growth and fruiting were obtained by determining the yields of mycelium in terms of dry weight.

In the case of cultures on liquid media, this presented little difficulty. The mycelium was removed from the culture vessel, rinsed free from nutrients with cold distilled water in a Gooch crucible (no significant loss of mycelium resulted from this method), and then placed in a tared aluminium foil cup. Two days at 70°C . was sufficiently long to dry the quantities of mycelium obtained to constant weight. The cups were then weighed on ^a mechanically weight-loaded precision balance accurate to 0.1 mg.

With colonies grown on agar media it was first necessary to free the mycelium from the substrate. Hall (1933) describes a method in which she treated the culture with dilute hydrochloric acid, and then heated it to melt the agar. The pre-treatment with acid was omitted in this work. The technique adopted was to add an excess of hot water to the culture and then to heat it in a steamer at 100°C for 20 to 30 minutes.

It was appreciated that this treatment was liable to result

in a loss of weight through soluble materials escaping through cell walls made permeable by killing, but this loss, and therefore the weight obtained was assumed to be proportional to the true dry weight of the mycelium.

The magnitude of the loss in weight of the mycelium by this treatment was assessed in the following way. Twelve mycelia produced in liquid culture were dried in desiccators to constant weight, and then soaked in cold water overnight. They were then immersed individually in three changes of boiling water for a total time of about 3 minutes and redried to constant weight. The mean loss was 5.5% of the initial dry weight. The same mycelia were resoaked and heated for 2 hours in water at 100°C. After this treatment, the mean loss was 12%. This would be equivalent to a loss of 6-8 mg. in the weights of mycelia of the order obtained in most of the experiments, i.e. 50-70 mg. The loss would probably be more nearly half this magnitude with the treatment normally applied. This error should be borne in mind in considering the significance of total weights given in some of the following experiments, where true dry weights of sporophores are added to dry weights of heated mycelia.

Yields of sporophores were, as indicated above, recorded gravimetrically. This method was found to be the most satisfactory, since replicates sometimes differed quite considerably in number and size of sporophores, yet in terms of total dry weight of sporophore tissue agreed closely. In certain cases, further data qualifying fruiting are presented. No difficulty

was experienced in separating fruit-bodies from the vegetative mycelium, even in the case of sporophore primordia.

The aluminium foil weighing cups which were used had a number of advantages, viz. they were not hygroscopic; their weight was about the same as the specimens which were weighed, and accuracy in weighing was therefore increased; and they could be used repeatedly, although it was necessary to reweigh them each time.

In the statistical analysis and presentation of data, methods set out by Paterson (1939, p. 31 et seq.) were used.

Further techniques are described in appropriate places in the text.

C. EXPERIMENTAL RESULTS.

I. Nutritional Factors.

a). Carbon.

(1) Sources of carbon.

The choice of substances to be tested was governed partly by a consideration of the sort of materials likely to occur in horse dung, the natural substrate of this organism, and partly by a desire to compare the responses of this isolate with those described by Voderberg (1948) for C.lagopus.

The substances chosen were:-

Monosaccharides:	Fructose, Galactose, Glucose.
Disaccharides:	Lactose, Maltose.
Polysaccharides:	Cellulose, Starch.
Alcohols:	Glycerol
Fatty Acids:	Oleic (as Na-Oleate)
Amino Acids:	dl- α - Alanine
Organic Acids:	Tartaric (as Na.K.-Tartrate, hydrated)

These were added at concentrations of 1% w/v. to a basal medium of 0.1% Alanine Agar, less glucose.

Since a low level of soluble carbohydrate might have been necessary to initiate growth upon media containing insoluble polysaccharides, both starch and cellulose were tested with and without the addition of 0.1% glucose.

In the preparation of the cellulose-containing media, the requisite quantity of filter paper was mechanically

macerated in distilled water in a blender, and after centrifuging and partial drying, was incorporated in the media.

All treatments were performed in triplicate. Media were sterilized by steaming on three successive days.

Since a number of treatments had by the 14th. day of incubation produced large crops of sporophores which would have deliquesced had they been left longer, fruit bodies from these were harvested at that date and the yields of mycelium determined after melting the agar. The more slowly developing treatments were left until the 20th. day before harvesting.

The results are presented in Table Ia.

A treatment with sucrose as the carbon source in the main experiment was spoilt by contamination. The usability of this carbohydrate was therefore investigated separately, and the results given in Table Ib.

Because it was virtually impossible to free the hyphae from fibres of cellulose, the dry weights as determined in such cases were valueless and are therefore omitted from the data (Table I a.).

Table Ia.

The Effect of Source of Carbon on Growth and Fruiting.

Source of Carbon	Period of Incubation, Days.	Mean Dry Weight, mg.			Mean Days to Fruit.
		Mycelium	Sporophores	Total Fungus [†]	
No Addition	20	10.8	0	10.8	-
Glucose	14	46.6	25.6	72.2	9.7
Maltose	"	33.6	20.9	54.5	7.3
Fructose	"	35.8	18.6	54.4	9.3
Starch + 0.1% Glucose.	"	36.7	15.9	52.5	7.7
Starch	"	26.7	10.2	36.9	8.0
dl- α -alanine	20	22.7	0	22.7	-
0.1% Glucose.	"	20.9	0	20.9	-
Glycerol	"	18.3	0	18.3	-
Na.K.Tartrate	"	14.2	0	14.2	-
Galactose	"	1.1	0	1.1	-
Lactose	"	1.1	0	1.1	-
Na. Oleate	"	0	0	0	-
Cellulose	"	-	12.0	-	12.0
Cellulose + 0.1% Glucose	"	-	16.5	-	12.0

† Significant differences between means of total fungus weight

at { p. = 0.05, 4.4 mg.
 { p. = 0.01, 6.0 mg.

N.B. Carbon sources in order of decreasing yield.

Table Ib.

The Effect of Sucrose on Growth and Fruiting.

Source of Carbon	Period of Incubation Days.	Mean Dry Weight, mg.			Mean Days to Fruit.
		Mycelium	Sporophores	Total Fungus ††	
No Addition Sucrose	28	16.8	0.3	17.1	-
	28	18.6	0	18.6	-

†† Difference between means of total fungus not significant
(p. = > 0.9)

Assessed visually, growth on cellulose media was good both with and without additional glucose.

It is seen from Tables Ia. and Ib. that the sources of carbon tested fall into three main categories, viz.

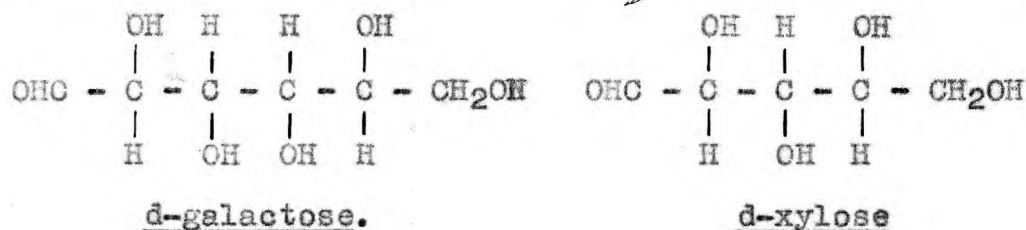
- (i) those which are readily usable, leading to good vegetative growth and fruiting, and which are all carbohydrates of various types.
- (ii) those which the fungus is able to use to a very limited extent or not at all, none supporting fruiting.
- (iii) those which actually inhibit growth. The presence of galactose and lactose in this group is particularly interesting.

The ready utilization of starch and cellulose was confirmed by repeating the appropriate parts of the above experiment. An iodine test on starch-containing media on which C.lagopus had grown revealed the absence of starch beneath and immediately around the colony.

An inhibition of radial growth by galactose similar to that noted above was recorded by Page (1952) for a species of Pilobolus. It is not easy to visualize the mechanism of such an inhibition by a hexose sugar, and in order to secure more data on this point, the effects of galactose and of chemically related sugars were studied further.

These additional sugars were:-

- (i) Xylose - a pentose sugar in which the configuration of part of the molecule is similar to that in galactose.



- (ii) Lactose - D.Glucose-4-βD. galactopyranoside, for which some data is already forthcoming.
- (iii) Raffinose - 6-αD. galactopyranosyl-αD. glucopyranosyl - βD. fructofuranoside.

Other closely related compounds were not readily available commercially. The above, and galactose, were incorporated in 0.1% Alanine Agar (less glucose) at concentrations of 1%, and in a second series glucose at 0.5% was added to each. This was in order to determine if any inhibition that might be produced could be offset by the presence of a readily utilized carbohydrate.

The four carbohydrates to be tested were sterilized by passing through a Seitz filter. The filtrates were then added

aseptically to the basal media which had previously been steamed on three successive days. Treatments were in quadruplicate.

Records were kept of radial growth and appearance. On the 15th. day of incubation, the sporophores, where present, were dissected away, and their dry weights and also those of the mycelia after freeing them from the agar were determined. The data are presented in Table II and Fig. I.

Table II.

The Effect of Galactose and Allied Sugars on Growth and Fruiting.

Additional Source of Carbon.	Without Glucose				With 0.5% Glucose.			
	Mean Dry Weight (mg.)			Days to Fruit	Mean Dry Weight (mg.)			Days to Fruit
	Myc.	Sporoph.	Total †		Myc.	Sporoph.	Total †	
Galactose	1.7	0	1.7	-	65.8	0 (+)	65.8	5.5
Lactose	1.6	0	1.6	-	27.5	0	27.5	-
Raffinose	11.2	0	11.2	-	24.5	20.9	45.4	6.5
Xylose	20.5	23.7	44.2	7.0	93.4	0.6	94.0	7.3
Nil	10.2	0	10.2	-	25.1	19.0	44.1	6.8

† Significant differences between means of total fungus weight.

at { $p = 0.05$, 4.0 mg.
 $p = 0.01$, 5.4 mg.

(+) indicates fruiting commenced, but was negligible.

It is clear from the table that the inhibitory properties of galactose and lactose were not shared with either raffinose or xylose. The latter was in fact readily utilized, whilst the former neither stimulated nor inhibited growth. The addition of glucose surprisingly led to galactose becoming stimulatory,

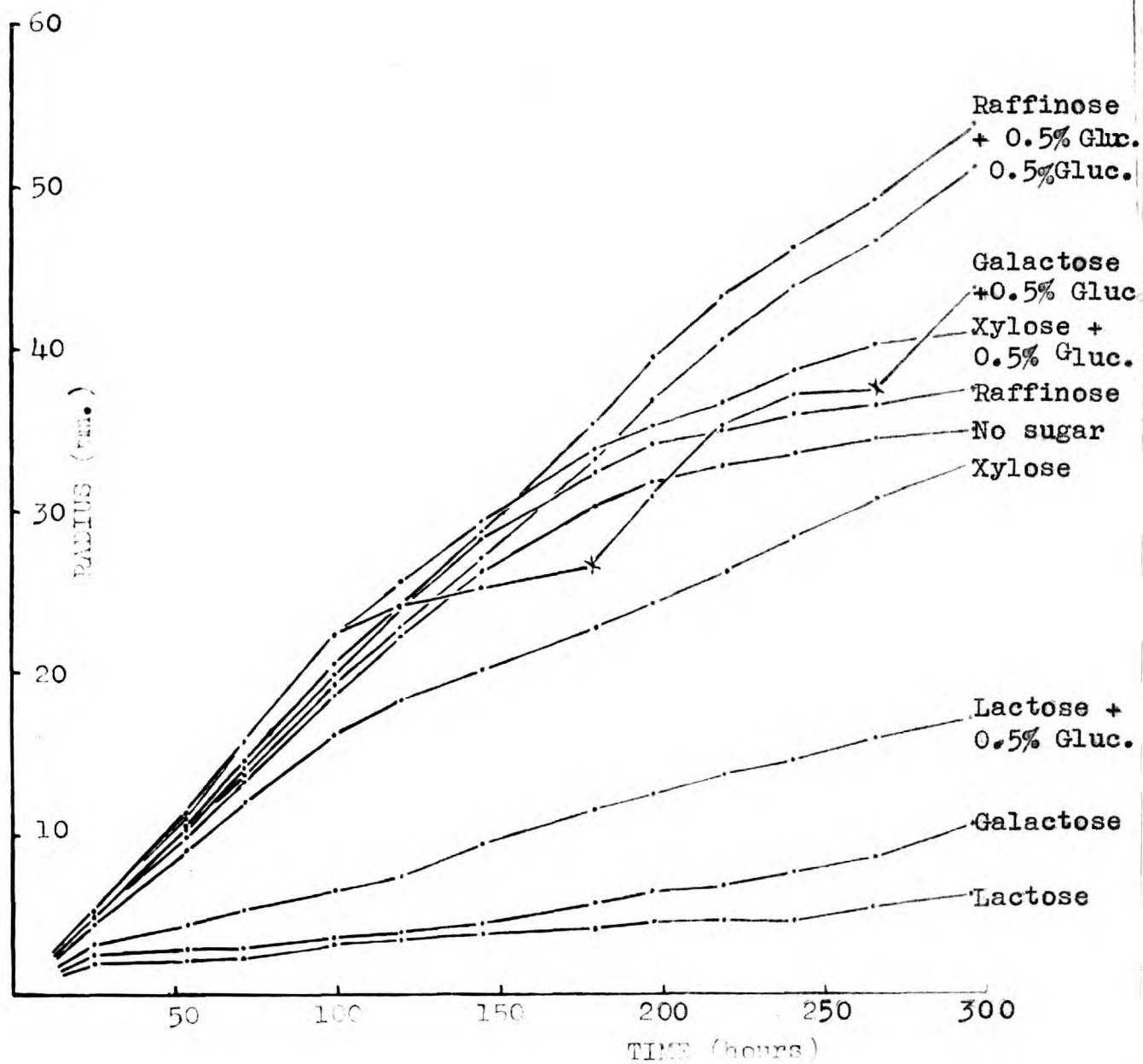


Fig. 1. Growth Rates of Coprinus lagopus with different Carbon Sources.

(-x- denotes the formation of a zone)

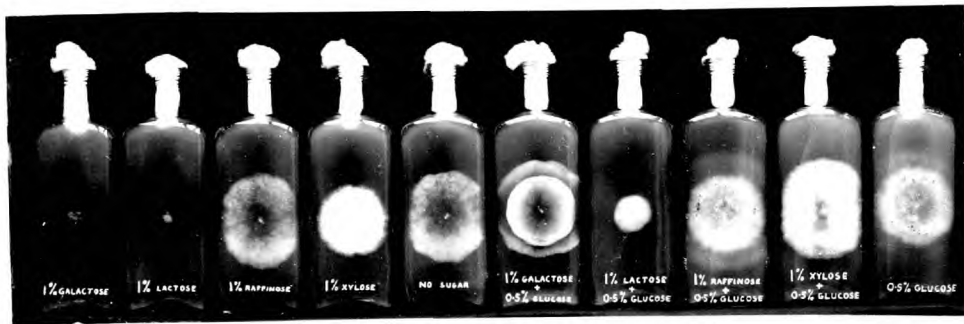


Plate I. The effects of galactose and allied sugars on growth and fruiting of Coprinus lagopus (10 days old).

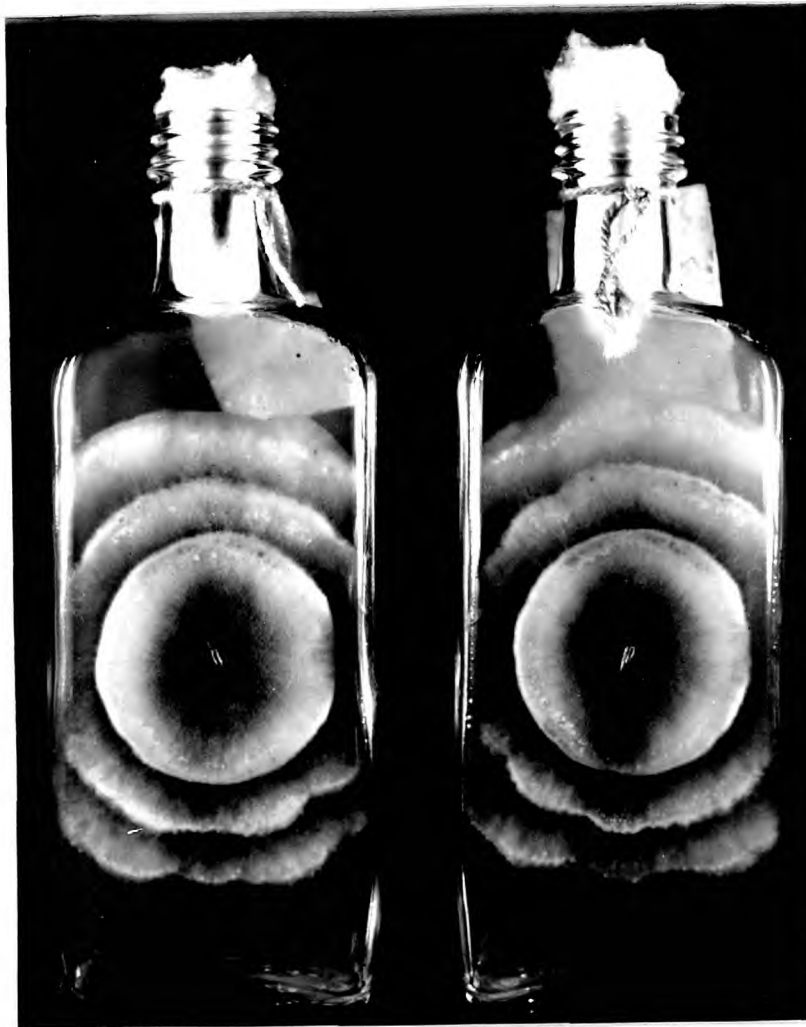


Plate II. The growth of Coprinus lagopus on 1% galactose - 0.5% glucose medium (15 days old).

with the growth of the colony adopting a pronounced zonation (see Plate I and II). This zonation was reflected in the rate of radial growth (see Fig. I). Lactose retained some inhibitory properties in the presence of glucose, when the growth although dense was very restricted in radial spread. Once again the fungus was unaffected by raffinose, while xylose supported a large amount of vegetative mycelium.

It is of interest to note the apparent antagonism between vegetative and reproductive growth which was well displayed in this experiment when glucose was added to medium containing xylose. The addition tremendously increased vegetative growth yet, greatly reduced fruiting. However this was not always displayed, as was shown by the results for "Nil" and "Nil plus 0.5% glucose", where both vegetative growth and fruiting increased as a result of adding glucose. This will be discussed later.

The zonation on galactose-glucose medium was produced by the progressive intensification of the density of mycelial growth, culminating in the laying down of primordia just within the margin of the colony, followed almost immediately by an abrupt change to a very thin, rapidly spreading vegetative mycelium. Since this change followed so soon after primordia were formed, it was thought possible that fruiting might be a direct response to conditions in the medium and the subsequent decrease in density of vegetative growth the consequence of this fruiting. A similar sequence of changes had been observed in colonies grown upon other media (see, for example, the

"0.5% glucose" and "0.5% glucose plus raffinose" treatments in Plate I). The following experiment was carried out to test this hypothesis. Advantage was taken of results from preliminary work which indicated that fruiting could be at least retarded by withholding light.

Twelve cultures were set up on 1% Galactose-0.5% Glucose - 0.1% Alanine Agar in medical flats; four were kept in continuous light and the remainder in continuous darkness. On the 12th. day, four of the latter were brought into the light and compared with the controls. The other four were examined on the 16th. day.

It was found that in all cases the vegetative mycelium was prominently zoned, but in only those cultures in continuous light were there any sporophore primordia. The zonation of the vegetative mycelium was then independent of primordium formation.

Although these cultures were kept for two months, none of the primordia developed into more than minute, very stunted sporophores.

ii) The effects of different glucose concentrations.

Various initial concentrations of glucose up to 6% were added to a basal medium of 0.1% Alanine Liquid Medium (less glucose). The media were adjusted to pH 8.0 by the addition of sodium hydroxide solution and then sterilized by steaming on three successive days. All treatments were in quadruplicate. After 15 days' incubation, the yields of vegetative mycelium

and of sporophore tissue were determined. The final pH of the media were also recorded (Table III).

Table III.

The Effects of different Initial Concentrations of Glucose on Growth, Fruiting and pH Drift in Liquid Culture.

	Initial Concentration of Glucose (%)									
	0	0.25	0.5	0.75	1.0	2.0	3.0	4.0	5.0	6.0
Dry wt. (mg.)										
Mycelium	0.6	19.5	29.6	48.9	74.6	81.0	79.2	64.0	52.8	41.5
"Sporophores	0	5.7	11.1	5.5	2.1	0.2	0	0	0	0
" Total	0.6	25.2	40.7	54.4	76.7	81.2	79.2	64.0	52.8	41.5
Final pH	7.79	7.75	7.47	6.88	7.00	6.87	6.79	6.72	6.76	6.63

The maximal yield of vegetative mycelium was at an initial concentration of 2-3% glucose, while that of sporophores was at only 0.5% glucose. Increases in concentration above this latter optimum led to a reduction in the number of replicates which actually began to fruit as well as to a diminution of the yield of the individual fruiting replicates. This reduction was at the expense of the development of the individual fruit body as well as of the number of fruit bodies developing. The numbers of replicates failing to fruit at supra-optimal concentrations of glucose were one at 0.75%, two at 1%, three at 2% and 3%, and all four at 4% and higher. In the table, these non-fruited replicates were included when calculating the mean yields of sporophore tissue.

The changes in reaction of the media after growth of the fungus for 15 days were not very large, but were clearly

greatest in those with highest initial glucose concentration.

The effects of different initial glucose concentrations were also studied in a basal medium of 0.1% Alanine Agar (less glucose). After adjusting the pH to 8.0, so that conditions were comparable in this respect with those in the previous experiment, the media were sterilized by steaming on three successive days. Treatments were in quadruplicate. The cultures were incubated for 17 days. As well as recording the yields of fungus, measurements of radial growth were made and the ages at which fruiting began were noted. The results are given in Table IV and Fig. II.

Table IV.

The Effects of different Initial Concentrations of Glucose on Growth and Fruiting upon 0.1% Alanine Agar.

	Initial Concentration of Glucose (%)									
	0	0.25	0.5	0.75	1.0	2.0	3.0	4.0	5.0	6.0
Dry Wt. (mg.) Myc.	5.5	14.7	19.3	21.7	34.8	107.6	111.7	75.2	34.4	12.7
" Sporoph.	0	9.3	15.8	22.0	22.8	0.2	0	0	0	0
" Total	5.5	24.0	35.1	43.7	57.6	107.8	111.7	75.2	34.4	12.7
Days to Fruit.	15->17	9.3	9.3	9.0	10.3	13.8	14->17	>17	>17	>17

(N.B. "15->17" days to fruit means one or more replicates commenced fruiting on the 15th. day, but some were still sterile on concluding the experiment on the 17th. day. This convention is used in later tables also).

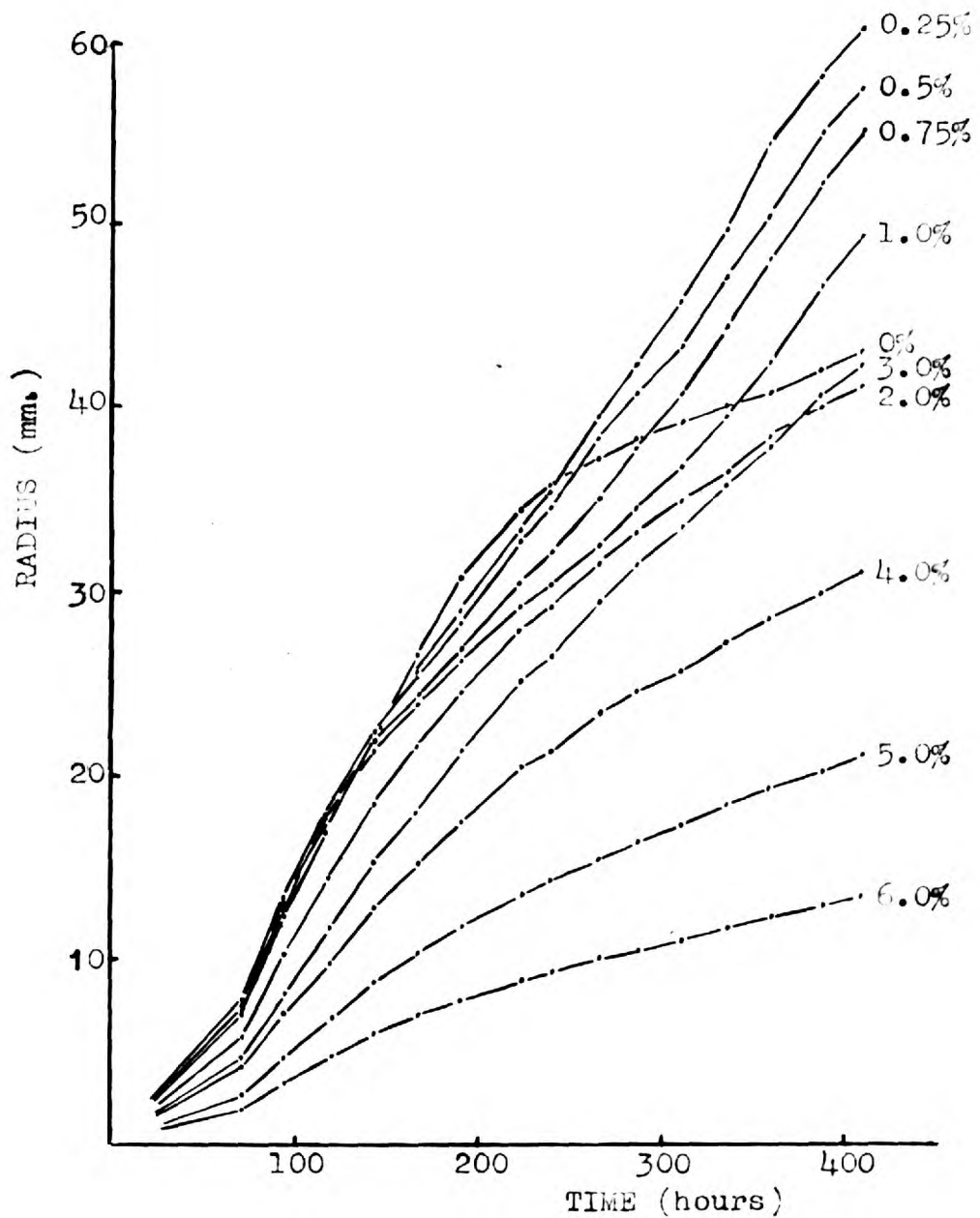


Fig. II. Growth Rates of Coprinus lagopus with different concentrations of Glucose.

The relationships between yields of vegetative mycelium and of sporophores and the initial concentration of glucose were similar to those which emerged from the previous experiment. The optimum for fruiting in this case was however a little higher than that found in liquid culture.

The rate of radial growth fell as the initial concentration of glucose was increased. The growth rate with 0% glucose, which was at first the greatest, diminished rapidly after about the 10th. day.

The sporophores produced at 0.75 and 1% initial glucose concentrations included mature specimens; those at lower concentrations were immature and smaller. Above 1% glucose, none had progressed beyond the primordial stage.

The irregularity of fruiting at supra-optimal concentrations was rather less marked in this experiment than it was in liquid cultures. Primordia were present in all replicates at 2% glucose; at 3% only one replicate fruited, with a negligible quantity of sporophore tissue; at 4% and above no primordia were formed.

Since high initial concentrations of glucose delayed the appearance of the first primordia, it might be suggested that the low yields of sporophore tissue obtained in such cases were the result of harvesting the growth at too early a stage of development. This possibility will be discussed later (p.102)

The above shows that high concentrations of glucose delayed and reduced fruiting. Similar results were obtained for

Chaetomium globosum by Basu (1947) who showed that fruiting coincided with the depletion of reducing sugar in the medium to a low level which was independent of initial concentrations of glucose over the range 0.1 to 2.0%. However the same worker showed that Memnoniella echinata behaved differently; fruiting was not delayed by increases in glucose concentration from 0.5% up to 1.5%, nor was the level of reducing sugar in the medium reduced to anything like a uniform level at the time of fruiting.

In view of these results, it was decided to determine the quantity of reducing sugars present in the medium at the onset of primordium production by C. lagopus.

A series of cultures was set up with initial glucose concentrations up to 5% in a basal medium of 0.1% Alanine Liquid Medium (less glucose). Each treatment was in quadruplicate. The cultures were incubated until primordium production commenced, when the dry weights of fungus were determined and the residual concentration of reducing sugar in the medium estimated by the method of Shaffer and Somogyi (1933). Because a single primordium sometimes appeared prematurely, for the purposes of this experiment, the initiation of fruiting was taken as the appearance of ten primordia visible to the naked eye. Cultures which failed to fruit were harvested after 55 days, and the sugar concentration estimated. The results are presented in Table V.

Table V.

The Weight of Mycelium and the Concentration of Reducing Sugars in the Medium at Onset of Fruiting.

Initial Glucose		Mean Days to Initiation of Fruiting.	Mean Residual Glucose mg./100 ml.	Mean Dry Wt. mg./culture.
mg./100 ml.	Approx. %			
0	0	> 55	0	2
245	0.25	8.8	60	25
475	0.5	8.5	285	24
715	0.75	7.3	505	25
975	1	9.0	535	43
1945	2	11.0	1150	80
2920	3	17->55	1420	116
3705	4	17->55	2755	120
4775	5	17->55	3180	135

† 2 or 3 replicates failed to fruit within duration of experiment.

The variation between replicates in the residual concentrations of reducing sugar and in the dry weights of fungus was fairly large because the differences normally experienced in liquid culture were exaggerated by the different periods of incubation. Nevertheless, it was perfectly clear that although fruiting was delayed by high concentrations of glucose, its initiation did not coincide with the attainment of a critical level of sugar in the different media. Indeed, in a number of cases primordia appeared at concentrations higher than the initial concentrations in other treatments which still needed seven or more days to fruit.

It will be noted that the dry weight of mycelium at the time fruiting commenced was also not nearly the same in the

different treatments.

b) Nitrogen.

(1) Sources of nitrogen.

The substances tested included representatives of the three main classes of nitrogen sources, viz., nitrate-, ammonium-, and organic-nitrogen. Of the latter group, substances of increasingly complex chemical structure were tested, including amino acids, an amide and bacteriological peptone. These substances were added to a basal medium of:

Glucose	1%
K ₂ HPO ₄	0.2%
MgSO ₄ ·7H ₂ O	0.02%
Aneurin HCl.	50 γ/100 ml. medium
Agar.	2%

The concentrations were such that the amounts of nitrogen present in the different treatments were the same and equivalent to M/30 sodium nitrate. In the case of peptone, the manufacturer's figure of 14.5% nitrogen was used in obtaining the appropriate concentration.

Since the utilization of nitrogen often leads to changes of pH of the medium, and preliminary work had indicated that acid conditions had an adverse effect upon the growth of Coprinus lagopus, each nitrogen source was tested alone and with 1% calcium carbonate. The latter prevented the reaction of the medium falling below pH 7.4.

All treatments were in quadruplicate. Media were

sterilized by autoclaving; the glucose was treated separately and added aseptically afterwards. The dry weights of vegetative mycelium and sporophores were determined on the 12th. day of incubation. Mycelia from media containing calcium carbonate were, after being freed from the agar, quickly rinsed in dilute (approx. 0.5N) hydrochloric acid to remove adherent chalk. The results are presented in Table VI with other data on radial growth and on age at onset of fruiting.

Table VI.

The Effect of Source of Nitrogen on Growth and Fruiting.

Source of Nitrogen	Without 1% CaCO ₃					With 1% CaCO ₃				
	Mean Dry Weight (mg.)			Days to Fruit	Mean Diam. on 9th day	Mean Dry Weight (mg.)			Days to Fruit	Mean Diam. on 9th day
	Myc.	F.B. †	Total ††			Myc.	F.B. †	Total ††		
No addition	8.4	0	8.4	-	72.1	13.9	0	13.9	-	73.3
Sodium nitrate	10.8	0	10.8	-	61.8	11.8	0	11.8	-	66.0
Ammonium chloride	23.4	0	23.4	-	70.5	35.2	19.3	54.5	9.5	79.1
Ammonium tartrate	21.5	0	21.5	-	64.6	35.5	0	35.5	-	63.4
Ammonium nitrate	25.2	0	25.2	-	74.5	20.1	50.2	70.2	9.0	83.8
Glycine	10.4	0	10.4	-	27.3	9.2	0	9.2	-	28.6
Alanine	33.9	0	33.9	10-12	65.8	32.1	0	32.1	12-12	62.1
Asparagine	16.7	0	16.7	-	38.8	12.6	0	12.6	-	38.8
Peptone	36.2	25.0	61.2	9.5	68.8	14.6	0	14.6	-	35.0

(Continued on next page).

Key to Table VI.

† F.B. = yield of fruit-bodies.

†† Significant difference between mean total dry weights

at $\begin{cases} p. = 0.05, & = 7.5 \text{ mg.} \\ p. = 0.01, & = 9.8 \text{ mg.} \end{cases}$

Adopting the total yield of fungus as an index of usability of the nitrogen source, it was evident that the fungus was able to make little or no use of nitrate-nitrogen. Good growth was made upon media containing ammonium salts although there was no fruiting. The utilization of each source of ammonium-nitrogen was significantly increased by the addition of calcium carbonate, and fruiting then became prolific with two of the three ammonium salts. The usability of the three salts differed highly significantly ($p = < 0.01$) in the buffered media. Of the organic sources, alanine and peptone were readily used. The reduced growth on the addition of calcium carbonate to the peptone medium was surprising.

Although with the criterion here used, the data indicated that glycine and asparagine were not usable, it must be mentioned that the growth of C.lagopus with these sources of nitrogen was very dense, although restricted in diameter (see Table VI). This contrasts with the control treatment in which, although the yield was much the same, the diameter was approximately double, and the mycelium very sparse.

The negligible fruiting with alanine in this experiment

was unexpected, for normally with this fruiting commences on or about the 10th. day.

The experiment was repeated, except for the treatments with glycine and peptone, and the above results confirmed.

The usability of urea was tested separately, both with and without the addition of calcium carbonate, and was found in both cases to support a moderately good growth of mycelium, but there was no indication of any tendency to bear fruit-bodies during the 12 days over which observations were made.

Since the adverse effect of calcium carbonate on the utilization of peptone was unexpected, it was further investigated in the following experiment.

Peptone media, with and without calcium carbonate, were prepared as above and sterilized by autoclaving. In a similar series the peptone was autoclaved separately and added aseptically afterwards. Each treatment was in quadruplicate. The results are given in Table VII.

Table VII.

The Effect of Calcium Carbonate and Method of Sterilizing on Peptone Utilization.

	Mean Dry Weight (mg.)	Without CaCO_3	With CaCO_3	Aggregate Total Mean
Peptone autoclaved separately.	Mycelium	30.7	32.2	-
	Sporophores	21.3	0	-
	Total	52.0	32.2	42.2
Peptone not autoclaved separately.	Mycelium	33.1	36.1	-
	Sporophores	28.2	3.6	-
	Total	61.3	39.7	50.5
Aggregate Total Mean.		56.6	36.0	

Significant difference between mean aggregate Total Yields at

{ $p=0.05$, 15.0 mg.
 $p=0.01$, 26.9 mg.

Significant difference between Mean Aggregate Total Yields at { $p = 0.05$, 9.8 mg.
 $p = 0.01$, 13.0 mg.

The interaction of the presence of calcium carbonate and the method of sterilizing was not significant. However the mean aggregate effect of calcium carbonate on total yield was significant ($p = < 0.01$), and confirmed the result of the previous experiment, although the effect was relatively less marked than before. It was also reflected in the yields of sporophore tissue. The mean aggregate effect of autoclaving the peptone with the rest of the medium or separately was not significant, which suggested that the adverse action of calcium carbonate was not caused by its reaction with peptone during autoclaving.

ii) The effect of different alanine concentrations.

The initial concentration of dl- α -alanine was varied within the limits 0 and 0.5% in a basal medium of:-

Glucose	1%
K ₂ HPO ₄	0.2%
MgSO ₄ .7H ₂ O	0.02%
Aneurin HCl	50 μ /100 ml.

- in distilled water.

The reactions of the medium was adjusted to pH 8.0, and the medium then sterilized by steaming on three successive days. This adjustment was necessary in view of the slight variations in pH resulting from the use of different quantities of alanine in the media. All treatments were in quadruplicate. After 16 days' incubation, growth was assessed, and the pH and reducing sugar content of the media determined. The data are presented in Table VIII.

Table VIII.

The Effects of different Initial Concentrations of Alanine on Growth, Fruiting, Glucose Consumption and Final pH of the Medium.

		Initial Concentration of Alanine, (%)								
		0	0.01	0.05	0.075	0.1	0.2	0.3	0.4	0.5
Mean Myc. †		1.0	22.5	77.3	99.1	73.9	84.6	86.9	65.3	78.0
Dry Wt. Sporoph. (mg.)		0	0	0.6	0.2	7.2	11.6	14.4	14.0	14.5
Total		1.0	22.5	77.9	99.3	81.1	96.2	101.3	79.3	92.5
Acid Drift (pH units)		1.19	1.19	1.21	1.31	1.19	0.86	0.48	0.19	0.09
Days to Fruit		>16	16->16	12->16	11.7	10.8	9.3	8.3	7.5	9.0
Glucose consumed per Culture (Init. Gluc. 200 mg.) ††		26	121	137	158	142	164	193	192	188

† Significant difference between mean Dry Weights of Mycelium

at $\begin{cases} p = 0.05, & = 16.9 \text{ mg.} \\ p = 0.01, & = 22.9 \text{ mg.} \end{cases}$

†† Significant difference between mean Glucose Consumptions

at $\begin{cases} p = 0.05, & = 25 \text{ mg.} \\ p = 0.01, & = 34 \text{ mg.} \end{cases}$

It is seen that the response in terms of mycelial growth to increases in alanine concentration was not uniform. Thus although increases in concentration up to 0.075% alanine led to significantly greater yields of vegetative mycelium, above

this level there was an abrupt fall which was highly significant statistically ($p = < 0.01$). This was followed by further but less pronounced increases and reductions which were not significantly higher or lower. (The differences between yields of vegetative mycelium at initial alanine concentrations of 0.3 and 0.4% was significant in itself, but was not displayed when this experiment was repeated in essentials, as described below). The significant reduction at 0.1% coincides with the lowest concentration at which the crop of sporophores attained an appreciable weight. Nevertheless despite this tendency towards a reciprocal relationship, the total yield at 0.1% alanine was still less than that at 0.075 and 0.2% concentrations. This is discussed later (p. 109)

In considering glucose consumption in relation to increase in alanine concentrations, although the successive increases in consumption were not all individually significant, it is clear that there was a general increase in glucose consumption up to 0.3% alanine, at which level almost all of the available glucose (200 mg.) was used.

Such pH drift as did occur was unlikely to have had an appreciable effect on the growth of the organism.

It is also clear that with the possible exception of 0.5% alanine, increases in initial concentration hastened the onset of fruiting.

In order to verify the peculiar response of vegetative mycelium to alanine concentration, and to investigate the

influence of the latter upon radial growth, the experiment was repeated in precisely the same fashion, but with the media solidified with 2% agar. The results after 16 days incubation are presented in Figures III and IV.

Fig. III shows that the radial growth rate varied systematically as the cultures aged in a similar fashion at all concentrations of alanine. It rose rapidly to a high level, then fell, and ultimately increased once again. It will be noticed that although the growth rate was initially highest with the greatest concentrations of alanine, the picture was reversed later. The overall effect, which is shown in Plate III, was that at the 9th. and 13th. days there was relatively little difference in colony size between different treatments.

The onset of fruiting was accelerated by high levels of alanine, but bore no obvious relationship to the fluctuations of the growth rates (Fig. III).

It is interesting to note the presence of a fringe in which aerial mycelium was sparse in those cultures where fruiting had begun. This reduction in density of aerial hyphae was normal in fruiting cultures (Plate III), being produced immediately after sporophore production commenced. The density of new growth was subsequently restored.

The abrupt fall in yield of vegetative mycelium, in the previous experiment at 0.1% alanine, here occurred at 0.075% (Fig. IV), and, as before, was associated with a marked increase in the yield of fruit-bodies.

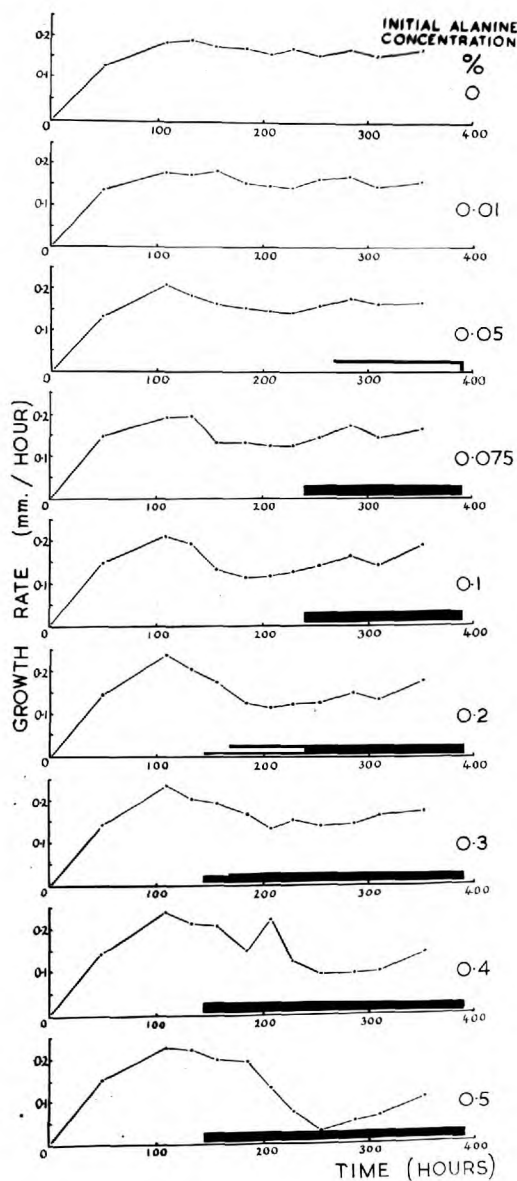


Fig. III. The Effects of different Initial Concentrations of Alanine on Rate of Radial Growth and Onset of Fruiting of Coprinus lagopus.

(Horizontal lines indicate period of fruiting in each of the four replicates of each treatment).

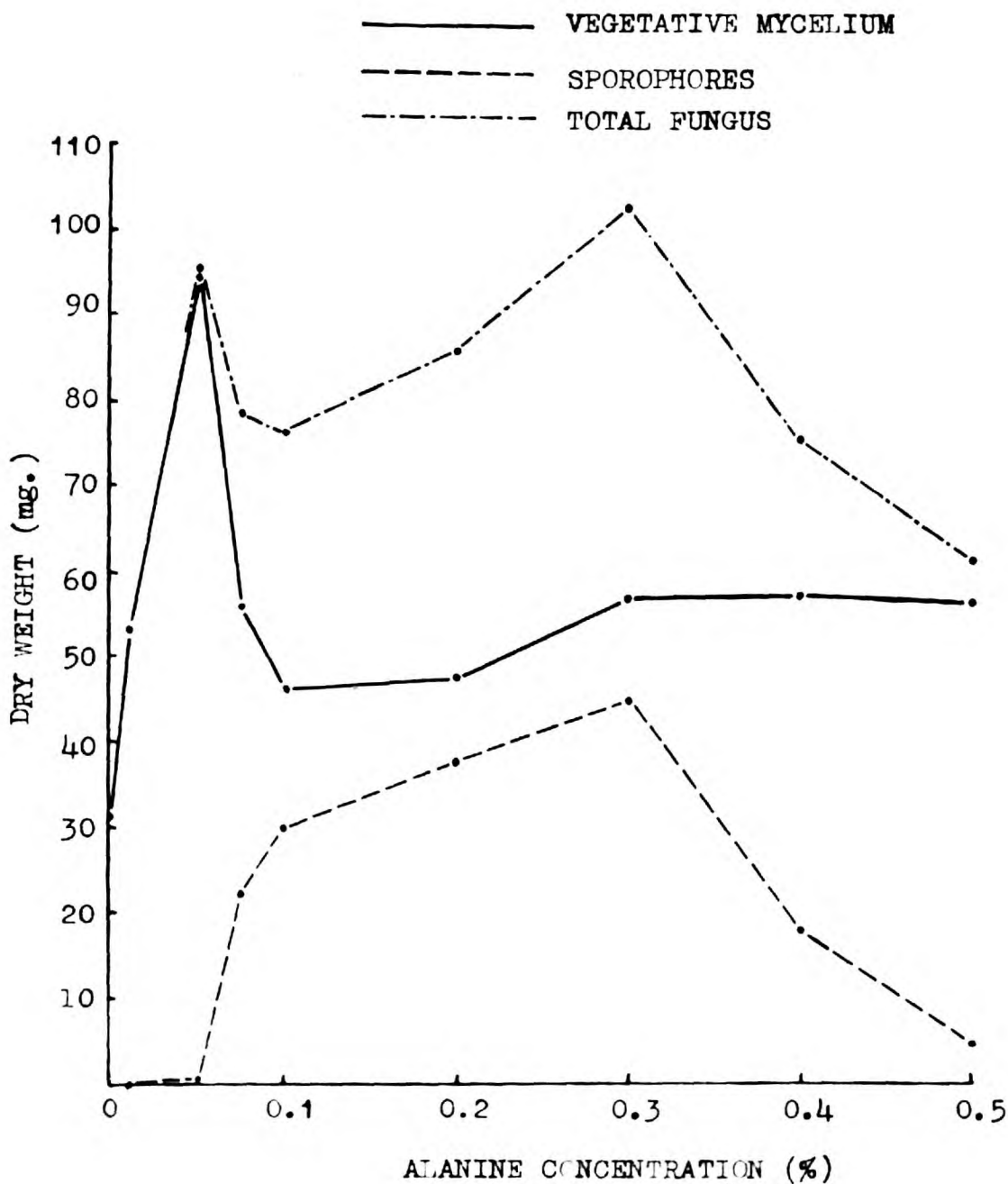


Fig. IV. The Effect of Initial Alanine Concentration in Agar Medium upon Mean Dry Weights of Yields of Vegetative Mycelium, Sporophores, and Total Fungus by Coprinus lagopus after Incubation for 16 Days.

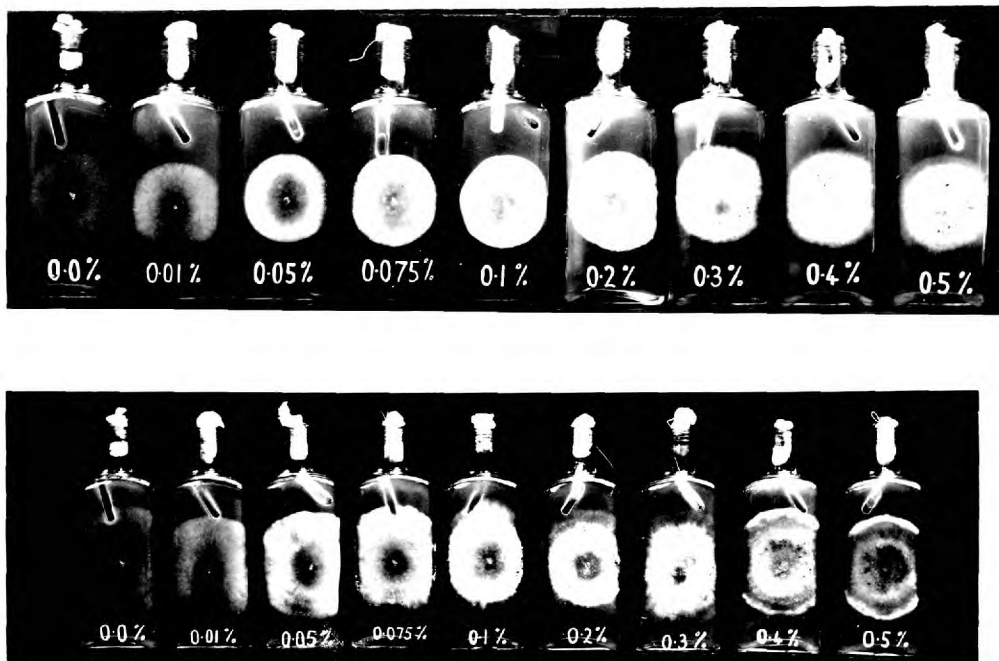


Plate III. The effects of different initial alanine concentrations on growth and fruiting of Coprinus lagopus.
Above: 9 days old. Below: 13 days old.
N.B. Reverse sides of cultures towards camera. Bases of fruit-bodies and initials appear as black dots.

The different yields of sporophores at different alanine concentrations are also illustrated in Fig. IV. The differences are in part attributable to the facts that all cultures were harvested at the same time and that fruiting was non-synchronous, as is shown by Fig. III which illustrates the times at which the individual replicates of treatments produced primordia. Thus the media with the lowest concentrations of alanine had failed to fruit within the incubation period. Each replicate of the series 0.05, 0.075, 0.1, and 0.2% showed respectively primordia, sporophores approaching maturity, and in the last two, mature sporophores. Using the blackish coloration of the pilei as an index of fertility (Borriss, 1934 a), the sporophores at or near maturity in these treatments were all fertile. Therefore within the range of alanine concentrations 0.05 - 0.2%, the variation in sporophore yield appears to be largely attributable to the differences in the times at which sporophore development began. At 0.3% alanine the same explanation applies in part, but the sporophores failed to mature properly. A few almost attained mature form, but judging by the cream colour of the pilei they were not fertile. The bulk of the yield of sporophore tissue was made up of a large number of small immature sporophores. At 0.4 and 0.5% alanine, the yields of fruit bodies were certainly not the result of non-synchronous fruiting. In both cases there was a very large number of primordia, and only at 0.4% alanine did any develop further, these showing premature autolysis. Thus although fruiting commenced earliest in those treatments

with high alanine concentration, the development of the individual sporophores was retarded or even arrested.

Possible explanations of the low yields of vegetative mycelia in those cultures in which sporophore production was in the ascendant were either that growth of vegetative mycelium was reduced after fruiting began, or that there was a withdrawal of material from the vegetative mycelium into the developing fruit-bodies.

To investigate these possibilities, thirty-six cultures were established upon 0.1% Alanine Agar in 10 oz. medical flats. The inoculum in each flat was placed upon the medium half way along one side, thus permitting a long^{er} period of uninterrupted growth than was obtained in previous experiments.

Starting on the 9th. day of incubation, four samples were taken at random at daily intervals, and the yields of sporophores and of mycelium, after freeing from agar by steaming, were determined. The results are given in Table IX.

Table IX.

The Distribution of Growth during Sporophore Formation.

Age of Sample, (Days)	Mean Dry Weight (mg.)			Stage of Sporophore Development.
	Mycelium	Sporophores	Total	
8.9 (9th.)	26.0	0.2	26.2	Initiation of Primorida.
9.7 (10th.)	30.1	0	30.1	
10.9 (11th.)	31.6	3.9	35.5	Enlarging.
11.7 (12th.)	37.2	4.5	41.7	
12.7 (13th.)	22.0	19.3	41.3	Maturation.
13.7 (14th.)	30.9	17.5	48.4	
15.0 (15th.)	30.2	29.9	60.1	Deliquescence
16.1 (16th.)	39.3	13.7	53.0	
16.7 (17th.)	43.0	19.8	62.8	Second crop beginning.
Signif. Diff. between Means at { p. = 0.05 { p. = 0.01	7.2 9.7	5.7 7.7	7.3 9.9	

The highly significant increase in dry weight of sporophores from the 12th. to 13th. days coincided with a highly significant drop of the same magnitude in dry weight of mycelium. Whereas increments in total weight over two-day periods up to the 12th. day were significant, over the period from the 11th. to 13th. day, total weight showed no significant increase, and there was in fact virtually no change between the 12th. and 13th. days. A fall in total weight just short of acceptable significance coincided with the highly significant loss in weight by the sporophore crops through deliquescence after the 15th. day.

These results were confirmed in a similar experiment, reported under a later section (see Fig. VII).

c). Carbon-nitrogen ratios.

Cultures in triplicate were set up at each combination of 0, 0.05, 0.1, 0.2 and 0.4% alanine with 0, 0.5, 1, 2 and 4% glucose. The basal medium to which these combinations were added was:-

K_2HPO_4	0.2%
$MgSO_4 \cdot 7H_2O$	0.02%
Aneurin HCl.	50 μ /100 ml. medium.
Agar.	2%

The media were sterilized by steaming on three successive days, and after inoculation were incubated for 15 days. The recorded data are presented in Table X.

The previously described effects of either factor singly were again borne out. Of especial interest was the effect of different relative concentrations of glucose and alanine on yield of sporophores. Increased concentration of alanine led to otherwise inhibitory levels of glucose becoming favourable for fruiting, and also raised the glucose concentration at which fruiting was maximal.

The interaction was reflected in the ages at which fruiting commenced. The effect of glucose was normally to retard the date at which primordia appeared, even to the extent of preventing their appearance at high concentrations. Increases in initial concentration of alanine ultimately completely

Table X.

The Effects of Glucose-Alanine Ratio on Growth and Fruiting.

(a) Mean Dry Weights of Mycelium. (mg.)

Alanine (%)	Glucose (%)				
	0	0.5	1	2	4
0	10.5	33.6	36.7	27.4	14.4
0.05	11.4	37.0	75.7	105.4	59.6
0.1	11.5	33.6	48.8	141.7	84.2
0.2	20.5	44.5	49.2	127.0	61.4
0.4	19.5	41.8	65.1	92.5	45.5

(b) Mean Dry Weights of Sporophores. (mg.)

Alanine (%)	Glucose (%)				
	0	0.5	1	2	4
0	0.4	0	0	0	0
0.05	0.4	F. 12.6	2.4	0	0
0.1	0	F. 12.6	F. 26.1	1.7	0
0.2	0	S. 14.8	F. 34.5	18.3	0
0.4	0	S. 6.5	S. 16.9	1.8	0

Significant differences between Mean Sporophore Yields

at $\left\{ \begin{array}{l} p. = 0.05, \text{ 10.4 mg.} \\ p. = 0.01, \text{ 14.1 mg.} \end{array} \right.$

F. = fertile sporophores. S. = sterile sporophores.

(c) Mean Dry Weights of Total Fungus. (mg.)

Alanine (%)	Glucose (%)				
	0	0.5	1	2	4
0	10.9	33.6	36.7	27.4	14.4
0.05	11.8	49.6	78.1	105.4	59.6
0.1	11.5	46.2	74.9	143.4	84.2
0.2	20.5	59.3	83.7	145.3	61.4
0.4	19.5	48.3	82.0	94.3	45.5

(contd.)

Table X. Contd.

(d) Mean Radius of Colonies on 9th. day. (mm.)

Alanine (%)	Glucose (%)				
	0	0.5	1	2	4
0	38	35	35	32	20
0.05	30	36	34	34	24
0.1	35	38	35	34	22
0.2	36	38	37	31	20
0.4	38	38	35	28	18

(e) Mean Age of Cultures at Onset of Fruiting. (days)

Alanine (%)	Glucose (%)				
	0	0.5	1	2	4
0	11.3	-	-	-	-
0.05	13.7	8.3	10.7	-	-
0.1	-	7.0	9.0	11.3	-
0.2	-	6.0	6.0	6.7	-
0.4	-	6.0	5.7	6.0	-

" - " indicates no fruiting within duration of the experiment, i.e. 15 days.

overcame the delaying effect of glucose, although the effects of supra-optimal alanine concentrations were then manifest, the fruit-bodies failing to develop properly.

The adverse effects of high alanine concentration upon sporophore development were on the other hand offset by increasing the glucose concentration. Whereas sporophores of mature form were sterile at 0.2% alanine and 0.5% glucose, at 1% glucose they were, as judged by the colour of their pilei, fertile.

The restriction of radial growth of colonies by high

concentrations of glucose was seen at all levels of alanine.

Of the two treatments in which fruiting commenced with 0% glucose, replicates at 0% alanine produced one or two extremely small sporophores approximating to the "dwarf fruit bodies" of Buller (1922).

d) Accessory Growth factors.

(1) Growth factor requirements.

Leonian and Lilly (1938) reported that C.lagopus required aneurin for growth. The requirements of the isolate used in this work for this and other well known growth factors were therefore examined.

The effects of additional low dosages of yeast extract ("Yeastrel") and of agar were also examined.

The basal medium in this experiment was:-

Glucose	1%
Alanine	0.15%
K ₂ HPO ₄	0.1%
MgSO ₄ .7H ₂ O	0.05%

The nutrient solution was first adjusted to pH 8.0 and then steam sterilized on three successive days in medical flats, with the solutions of appropriate growth factors contained separately in small suspended tubes. Biotin, folic acid and calcium pantothenate were supplied from refrigerated solutions; the others were freshly prepared. Each was added where appropriate to give a final concentration of 50 γ/100 ml. medium, except for inosite which was used at 20 mg./100 ml.

Agar and yeast extract were incorporated where required

in the nutrient solution before sterilization.

After sterilization, the contents of the small tubes containing growth factors were mixed with the nutrients by tilting the flats. The final volume of medium in each case was 20 ml.

The results, assessed in the usual way after 18 days' incubation, are given in Table XI.

Table XI.

The Effect of Various Combinations of Growth Factors and Natural Extracts on Growth and Fruiting in 0.15% Alanine Medium.

Growth Factors.	Mean Dry Weight (mg.)		
	Mycelium	Sporophores	Total Fungus.
None	5.7	0	5.7
Aneurin	69.2	16.3	85.5
Biotin + above	62.5	19.3	81.8
Inositol + all above	59.6	16.1	75.7
Riboflavin + " "	58.1	24.9	83.0
Nicotinamide + " "	69.1	16.9	86.0
Ca. Pantothenate + " "	60.3	24.1	84.4
Folic Acid + " "	49.5	17.1	66.6
Pyridoxine + " "	54.8	19.0	73.8
0.1% Agar + 8 Growth Factors	62.8	24.0	86.8
0.05% Yeastrel + 8 Growth Factors.	69.2	15.5	84.7
Significant Difference between Means			
at { p. = 0.05	23.5	13	
{ p. = 0.01	31.6	17.5	

The data clearly demonstrate the need for an external source of aneurin. There was no significant difference in yield of either mycelium or fruit-bodies as a result of the

addition of other specific growth factors, or of agar or yeast extract in the concentrations used.

Aneurin heterotrophy was confirmed in liquid culture on the following medium in which the nitrogen source was inorganic:-

NH_4NO_3	0.133%
CaCO_3	1%
Glucose	1%
K_2HPO_4	0.2%
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.02%

The effect of yeast extract was also tested. The salts were contained in one solution, glucose and aneurin or yeast extract in another. These solutions were steam sterilized separately and then mixed aseptically. All treatments were in quadruplicate. The growth as recorded after 20 days is given in Table XII.

Table XII.

Effect of Aneurin and Yeast Extract upon Growth and Fruiting in 0.133% Ammonium nitrate Medium.

Added Substance	Mean Dry Weight. (mg.)		
	Mycelium	Sporophores	Total Fungus †
None	1.1	0	1.1
Aneurin, 50 γ /100 ml.	5.3	0.7	6.0
Yeastrel, 20 mg./100 ml.	5.0	1.0	6.0

† Significant difference between means of Total Dry Weights of Fungus

at $\left\{ \begin{array}{l} p. = 0.05, = 3.5 \text{ mg.} \\ p. = 0.01, = 5.1 \text{ mg.} \end{array} \right.$

Three out of four replicates fruited in treatments with aneurin, and with yeast extract. The sporophores in the aneurin media remained small and immature, while in two of the yeast extract replicates they developed into very small, but mature, fruit-bodies of the "dwarf-type". None of the replicates in the basal medium fruited.

Although aneurin significantly increased the yield of fungus upon this medium with inorganic nitrogen, the total quantity of fungus was very much less than that produced in comparable treatments on alanine medium (see Table XI), in spite of a longer period of incubation.

(11) The effects of different aneurin concentrations.

Having established that the isolate needed aneurin for growth, its reactions to various concentrations of this factor were studied in view of the findings with certain other fungi that vegetative and reproductive growth were respectively less and more specific in their nutritional requirements.

To this end, four cultures were set up at each of the following concentrations of aneurin in 0.1% Alanine Agar, viz., 0, 0.1, 1, 5, 25, 50 and 500 γ per 100 ml. of medium. The media were prepared with the K_2HPO_4 in one solution, and the rest of the nutrients plus the appropriate quantities of aneurin in another. After autoclaving, the two solutions were mixed aseptically. The results after 13 days' incubation are given in Table XIII.

Table XIII.

The Effects of different Aneurin Concentrations in 0.1% Alanine Agar on Growth and Fruiting.

Aneurin Concentration μ/100 ml.	Mean Dry Weight (m.g.)			Days to Fruit	Mean Radius 13th day. (mm.)
	Mycelium	Sporophores	Total †		
0	44.8	0	44.8	-	48.3
0.1	50.8	0	50.8	-	49.6
1	55.4	0 (+)	55.4	12→13	44.9
5	26.1	31.3	57.4	9	53.1
25	27.7	30.2	57.9	9.5	52.3
50	28.8	31.7	60.5	10	53.0
500	35.4	29.5	65.1	9.5	51.8

† Treatment effects not significant ($F = 2.52$, for $p. = 0.05$, $F = 2.63$).

Although the treatment effects upon total yield of fungus were not quite significant at $p. = 0.05$, the density of aerial mycelium as assessed visually was clearly decreased by reducing the aneurin concentration below 1 μ per 100 ml. Radial growth varied little. Fruiting showed a very marked response. At 1 μ per 100 ml., primordia formed in three of the four replicates, but their dry weight was negligible. All treatments above this concentration fruited in all replicates; below it, all remained sterile.

e). Organic acids.*

A number of workers have described stimulation of growth of various fungi by the inclusion of certain organic acids in the culture medium. Leonian and Lilly (1940) recorded that the ability of Phycomyces blakesleeanus to utilize ammonium nitrogen was increased by the presence of succinic acid.

From the previous experiments on the effects of growth factors on C.lagopus cultures in liquid media with alanine and with ammonium nitrate as nitrogen sources, it was clear that under these conditions the organic nitrogen source was the more readily usable. Accordingly the effect of a number of organic acids was tested upon both sorts of medium.

Six were chosen, viz. acetic, citric, fumaric, glutaric, maleic and succinic. Solutions of these acids or their salts were prepared and adjusted to pH 7.0 with sodium hydroxide solution. They were then incorporated in basal media of 0.1% Alanine Liquid Medium or 0.133% NH_4NO_3 + 1% CaCO_3 Liquid Medium, at final concentrations of M/100. The media were sterilized by autoclaving, the phosphate being treated separately and added later. Cultures were incubated for 17 days, and the total

* During the course of these investigations it was suspected that the vigour of the isolate when grown in liquid culture had declined. A comparison of the growth made under identical conditions over 16 day periods in September 1952 and August 1953 revealed that at the later date there was no fruiting and a 40% reduction in total yield of fungus. In other experiments late in the work, although fruiting did occur, it was delayed and erratic.

The experiments in which liquid cultures were used in section (e), (f) and (g) were all conducted after this decline had taken place.

yield of fungus, which in all cases was entirely vegetative, was then determined. Table XIV contains the results.

Table XIV.

The Effect of Organic Acids on Growth and Fruiting upon Alanine and NH_4NO_3 media.

Organic Acid.	Mean Total Dry Weight, (mg.)	
	Alanine	NH_4NO_3
None	61.9	23.2
Acetic	80.8	19.5
Citric	1.5	12.9
Fumaric	43.9	26.9
Glutaric	63.3	15.1
Maleic	33.4	20.4
Succinic	26.1	16.8

Significant differences between means of total dry weights.

at $\begin{cases} p. = 0.05, & 15.6 \text{ mg.} \\ p. = 0.01, & 21.1 \text{ mg.} \end{cases}$

In the ammonium nitrate series there were no significant differences between the control and other treatments. A significant increase was produced by acetic acid in the alanine medium ($p. = < 0.05$). Certain other acids actually depressed growth significantly (fumaric at $p. = < 0.05$; succinic, maleic and citric at $p. = < 0.01$).

It is clear that the basal alanine medium gave a much greater yield of fungus than did the ammonium nitrate medium (difference significant at $p. = < 0.01$).

f). Agar.

From experiments described previously it is clear that C.lagopus grew much better on 0.133% NH_4NO_3 + 1% CaCO_3 Medium when solidified with 2% agar than when it was liquid (compare Tables VI and XII). Robbins (1939) found that agar contained certain substances which stimulated growth and fruiting of Phycomyces blakesleeanus, and that these substances could be removed from agar by washing with aqueous pyridine or methyl alcohol, the washed agar then supporting a reduced growth. Agar extracts added to liquid media restored growth to a level approaching that on unpurified agar.

The growth of C.lagopus on washed agar was therefore investigated. Two methods of washing were adopted, the one using distilled water, the other 5% aqueous pyridine. Appropriate quantities of agar powder were suspended in these solvents for one or two days at $4^\circ\text{C}.$, and then precipitated by centrifuging at high speed. The precipitate was resuspended in fresh water or pyridine and the process repeated. The final precipitates of agar were washed in 95% ethyl alcohol and dried at $35^\circ\text{C}.$

The two preparations and the unpurified agar were separately added to 0.133% NH_4NO_3 + 1% CaCO_3 . - Medium at concentrations of 2% in quadruplicate. The mean radial growths of colonies grown upon each of these media are shown in Fig. V.

The rates of radial growth on both of the washed agars were considerably less than that on the unpurified.

The production of primordia on the unpurified agar medium

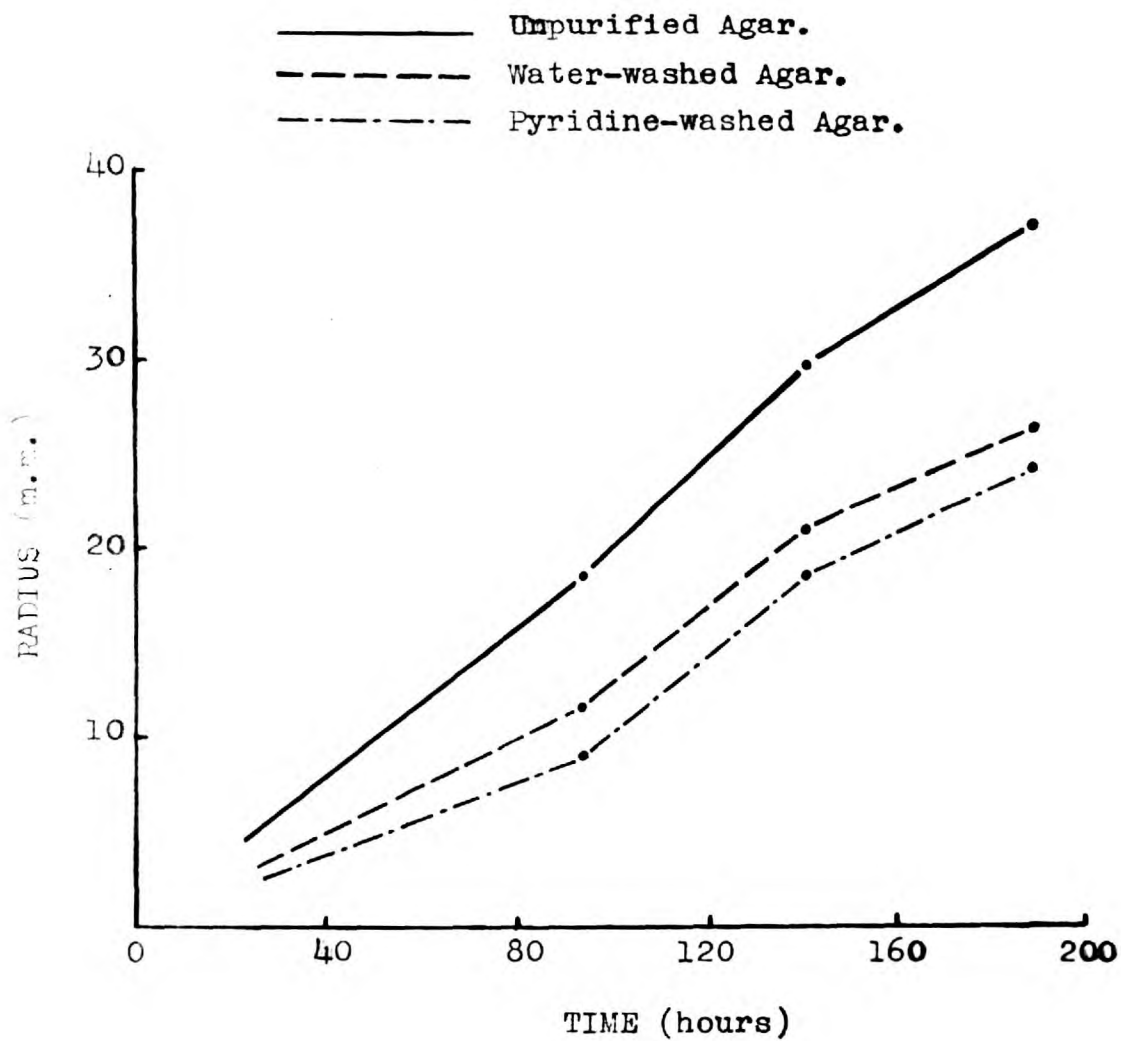


Fig. V. Radial Growth of Coprinus lagopus Colonies on Ammonium Nitrate Media prepared from Unpurified and Washed Agars.

began on the 7th. day of incubation, while the mean ages at onset of fruiting were 8.5 and 8.8 days respectively for water - and pyridine - washed agars.

From this experiment it was clear that agar contained some stimulatory substance which was removable by either pyridine or water.

The effect of agar extract upon growth in liquid culture was measured gravimetrically. An experiment similar to the above was conducted with additional treatments intended to indicate the nature of the stimulatory substance.

Six treatments were adopted: (i) 2% unpurified agar; (ii) 2% water-washed agar (prepared as described above); (iii) aqueous extract of agar and (iv) ashed agar, each added to the basal medium at concentrations equivalent to 2% unpurified agar; (v) unpurified agar at 0.3%, at which concentration a very weak and readily broken gel was formed; (vi) the basal medium alone, this being 0.133% NH_4NO_3 + 1% CaCO_3 -Liquid Medium.

The aqueous extract of agar was reduced to a convenient volume by evaporation under reduced pressure at 80°C. before adding it to the medium. The ashed agar was prepared by incinerating powdered agar in a crucible and dissolving the ash in a minimum of hydrochloric acid. The solution was adjusted to pH 7.0, at which pH it became slightly turbid, before adding it to the medium.

A similar range of treatments was set up using a basal medium of 0.1% Alanine Liquid Medium, for it had been noticed that even here agar affected sporulation.

In each of the two series of treatments the growth of the fungus upon the 2% unpurified agar was so much faster than in the other treatments that it was necessary to measure their dry weights at an earlier date, for otherwise the deliquescence of the large crops of sporophores would have resulted in misleading yield data. Therefore the cultures on the NH_4NO_3 and 2% unpurified agar medium were harvested on the 12th. day, and those on the alanine and 2% unpurified agar medium on the 14th. day of incubation. Table XV contains the data for all treatments, the rest of which were harvested after 17 days' incubation.

Mycelia formed upon agar media were freed by melting the agar. All mycelia were washed quickly with dilute (approximately 0.5 N.) hydrochloric acid - necessary in the case of the ammonium series to eliminate adherent calcium carbonate - so that the two series were more closely comparable.

Table XV.

The Effects of Agar and Agar Products on Growth and Fruiting on (a) Alanine, and (b) Ammonium nitrate Media.

(a) Alanine medium					(b) NH_4NO_3 medium			
Treatment	Mean Dry Weight (mg.)			Mean Diam. on 6th day (mm.)	Mean Dry Weight (mg.)			Mean Diam. on 6th day (mm.)
	Myc.	Sporoph.	Total		Myc.	Sporoph.	Total	
<u>Liquid Media:-</u>								
No Addition	61.9	0	64.4	28.9	23.2	0	23.2	27.5
+ Ashed Agar	65.5	0	65.5	28.6	44.5	0	44.5	27.1
+ Agar extract	107.5	2.6	110.1	37.9	79.1	0.7	79.8	44.6
<u>Solid media:-</u>								
2% Washed Agar	39.6	21.5	61.1	32.1	35.3	0	35.3	19.8
0.3% Unpurif. Agar	73.2	11.8	85.0	-	65.6	0	65.6	-
2% Unpurif. Agar †	28.2	31.9	60.1	50.7	17.5	45.4	62.9	53.0
Signif. Diff. between means at								
{ p. = 0.05	-	-	21.3	-	-	-	17.7	-
{ p. = 0.01	-	-	29.4	-	-	-	24.4	-

(† In both parts of the experiment this treatment was omitted from the statistical analysis through having had a shorter period of incubation).

It is clear that with ammonium nitrate, 2% unpurified agar greatly stimulated total growth of the fungus and fruiting. 2% washed agar was not significantly better than the basal liquid medium alone, and supported no fruit bodies. 0.3% agar supported greatly increased vegetative growth only. Similarly

agar extract gave a highly significant increase in vegetative growth of 244% above the control but produced negligible fruiting. The stimulation of ashed agar was also significant, but did not equal that of the aqueous extract.

With the alanine media, the only significant increase in total growth was produced by the addition of the agar extract to the basal liquid medium. As with ammonium nitrate medium there was little fruiting, amounting in this case to a few primordia in each replicate. Unpurified agar gave the best; here, however, the total yield of fungus was relatively low owing possibly to the shorter incubation period and the harvesting of the cultures whilst still actively growing.

The measurements of radial growth made on the 6th. day confirmed the results of the previous experiment, and showed that the aqueous extract of agar stimulated the radial growth of colonies in liquid media to which it was added.

When harvesting the cultures on unpurified agar in both series, it was noted that only large fruit-bodies or very small primordia were present. It was estimated that the dry weights of the latter would be very small. Therefore, ignoring these, it was possible to calculate the mean dry weights of individual mature or nearly mature sporophores from each treatment, as shown in Table XVI.

Table XVI.

The Effect of Nitrogen Source on the Number and Size of Sporophores Maturing in Culture.

Replicate	Alanine medium			NH ₄ NO ₃ + CaCO ₃ medium		
	Number of Large Sporoph.	Dry Wt. of Sporoph. Tissue (mg.)	Mean Individ. Dry Wt. (mg.)	Number of Large Sporoph.	Dry Wt. of Sporoph. Tissue (mg.)	Mean Individ. Dry Wt. (mg.)
a	5	29.6	5.9	2	51.5	25.8
b	6	41.7	7.0	2	43.3	21.7
c	4	34.6	8.3	2	36.7	18.4
d	6	21.5	3.6	4	50.2	12.6
Mean	5.3	31.9	6.1	2.5	45.4	18.2

On this basis it is apparent that the sporophores on the ammonium nitrate medium were approximately three times as large as those on alanine medium. Similar differences in size have been observed in other experiments with these media.

It was reported by Robbins (1939) that the differences in growth and fruiting of Phycomyces blakesleeana upon agar and liquid media became less as the cultures aged, the beneficial effect of agar being especially marked during early growth of the colonies.

To investigate whether Coprinus lagopus behaved similarly, certain treatments of the above experiment were repeated with a longer incubation period, viz., 22 days. Data are presented in Table XVII.

Table XVII.

The Effects of Purification of Agar and Aqueous Extracts of Agar on Growth and Fruiting after 22 Days Incubation.

Treatment	Mean Dry Weight (mg.)					
	0.1% Alanine Medium			0.133% NH_4NO_3 + 1% CaCO_3 Medium		
	Myc.	Sporoph.	Total Fungus	Myc.	Sporoph.	Total Fungus
2% Unpurified Agar	34.0	32.7	66.7	16.1	39.9	56.1
2% Washed Agar	48.1	20.8	68.8	27.2	35.9	63.0
Basal Liquid Medium	104.8	0	104.8	40.2	0	40.2
" + Agar Extract	69.2	25.1	94.4	79.6	0	79.6

All of the treatments on 2% agar bore mature and deliquesced sporophores. The only liquid medium bearing sporophores was that containing alanine and agar extract.

In the presence of alanine, the total growth was less with the solid than with the basal liquid media. It should be borne in mind, however, that there was some deliquescence of the sporophores on the solid media, and that some loss of weight probably resulted from the process of freeing the mycelium from the agar.

With ammonium nitrate, the basal liquid medium was still the least productive, but the increase through the addition of agar extract was relatively considerably less than that obtained previously after 17 days (98% as compared with 244%).

An effect of washing the agar was to retard the onset of fruiting from a mean of 9.0 days in the case of alanine, and 7.8 days for ammonium nitrate, to 13.5 days in both cases.

Nevertheless the sporophores still matured normally.

g). Trace elements.

During the progress of this work, it became apparent that the isolate was fruiting less readily in liquid culture than formerly. Smith (1948) described a similar loss in fertility with a number of species of Penicillium, and showed that it was associated with a change in the source of distilled water used in the preparation of media, and could be corrected by the addition of copper and zinc.

Since the source of distilled water was changed during the course of this investigation with C.lagopus, the possibility that trace elements were involved here also was investigated in the following experiment.

Copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) was added at 5 and 0.1 ppm., and zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) at 10 at 0.2 ppm. to 0.1% Alanine Liquid Medium. In each treatment, 1 ppm. of each of ferric sulphate, ammonium molybdate, manganese sulphate and calcium nitrate were also added. As a further treatment, the effect of ashed agar alone, equivalent to 2% fresh agar, was again examined. Treatments were in quadruplicate. The results after 21 days' incubation are presented in Table XVIII.

Table XVIII.

The Effects of Trace Elements and Ashed Agar upon Growth and Fruiting in 0.1% Alanine Liquid Medium.

Inorganic Addition	Mean Dry Weight (mg.)		
	Mycelium	Sporophores	Total [†]
None	90.4	0	90.4
Ashed Agar ($\equiv$ 2% fresh Agar)	102.8	0.7	103.5
5 ppm. CuSO_4 + 10 ppm. ZnSO_4 + 1 ppm. of other salts. }	92.5	0	92.5
0.1 ppm. CuSO_4 + 0.2 ppm. ZnSO_4 + 1 ppm. of other salts. }	85.1	0.1	85.2

† Treatment effects on Total Yields not significant.

($F = 3.64$; for $p. = 0.05$, $F = 3.71$).

The treatments did not cause any significant variation in total yield of fungus, nor did they affect the extent of fruiting, for in all treatments there were one to three replicates of the four with a number of small primordia. The previous observation that inorganic constituents of agar had no significant effect upon growth on alanine medium is confirmed.

h). Quantity of Substrate.

Voderberg (1949) reported that a certain minimum quantity of mycelium was a prerequisite for fruiting of Coprinus lagopus, this amount corresponding to that developing upon at least 1.5 g. of horse dung.

In order to investigate this effect, a number of discs of 0.1% Alanine Agar were prepared, having a uniform thickness,

but different diameters ranging from 7.5 up to 50 mm. These were made by pouring 20 ml. of sterilized medium into Petri dishes, each 90 ± 1 mm. in diameter, with flat bases. Discs of agar were then removed aseptically, after cutting with either sterile cork-borers, or, in the case of the larger discs, with a sterile scalpel, having previously drawn the outline of the discs on the bases of the plates with the aid of templates. Several discs of various sizes were placed in each of a number of sterile Petri dishes, and subsequently inoculated centrally. In all cases there were at least five replicates; the exact numbers are given in parentheses at the foot of Table XIX, in which the yields of vegetative mycelium and of sporophores at the time of maturation of the first fruit body on each disc are presented. The ages of the discs at the times of maturation are also given.

Table XIX.

The Influence of Quantity of Substrate upon Yields of Vegetative Mycelium and Sporophores at Maturity.

Diam. of Disc (mm.)	7.5	10	12.5	17.5	20	24	30	40	50
Mean Dry Vegetative Mycelium (mg.)	0.14 ± 0.024	0.23 ± 0.080	0.23 ± 0.030	0.54 ± 0.031	0.77 ± 0.030	1.56 ± 0.109	1.93 ± 0.092	3.58 ± 0.135	4.94 ± 0.349
Mean Dry Weight Sporoph. (mg.)	0.15 ± 0.022	0.78 ± 0.162	0.8 ± 0.083	1.84 ± 0.135	2.17 ± 0.074	3.08 ± 0.209	4.58 ± 0.309	8.91 ± 0.577	13.55 ± 1.245
Mean Days to Maturity	10.7	10.4	10.4	11.7	11.5	12.0	12.5	12.9	12.8
Number of Replicates	(5)	(6)	(10)	(15)	(15)	(14)	(8)	(6)	(5)

It was found that discs of all sizes fruited, although several of the smaller discs dried out before the sporophores matured.

The yields of vegetative mycelium and of sporophore tissue were more or less directly proportional to the volumes of the discs (Fig. VI). On all sizes of disc, the number of maturing sporophores was of the same order, viz. 1-4. Consequently, in general, the size of the individual mature sporophore was in relation to the quantity of substrate. The fruit-bodies on the smaller discs were comparable to Buller's "dwarf type".

Fruit bodies matured sooner on the smaller discs. This could have arisen by earlier fruiting or by more rapid maturation.

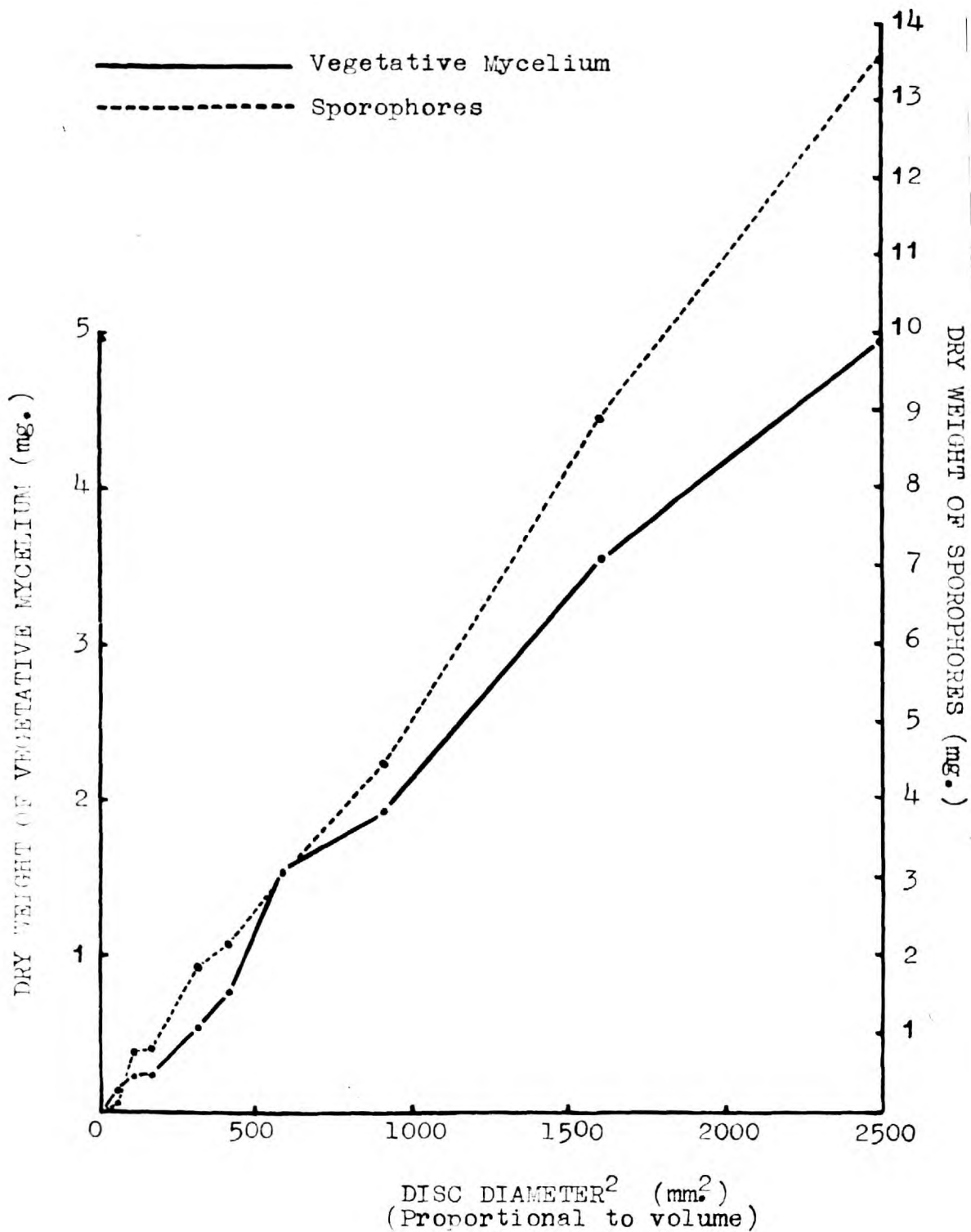


Fig. VI. The Effect of Quantity of Substrate on Yields of Vegetative Mycelium and Sporophores of Coprinus lagopus.

of the smaller sporophores. To determine which was the case, the above experiment was repeated, noting the ages of the discs at the time of the appearance of the first primordia and again at maturation of the first sporophores.

Table XX

The Influence of Quantity of Substrate upon Onset of Fruiting and Maturation of Fruit Bodies.

Diam. of Disc mm.		7.5	10	12.5	17.5	20	24	30	40	50
Days to P.P.†	Mean	8	7.1	7.5	8.7	9.4	9.4	11.3	13.3	14.2
	Range	6-10	7- 8	7- 8	7-10	8-11	9-11	10-13	11-15	12-15
Days to M.S.†	Mean	12	11.1	11.6	13.2	13.5	13.8	15.7	>16	>16
	Range	12	11-12	11-13	11-14	12-15	13-15	15-17	16->17	16->17
Days for Sporoph. to Mature		4.0	4.0	4.1	4.5	4.1	4.4	4.4	-	-
Number of Replicates		(6)	(12)	(13)	(13)	(14)	(10)	(7)	(10)	(6)

† P.P. = Primordium production.
M.S. = Maturation of Sporophores.

The results (Table XX) show that fruiting commenced soonest on small discs, and that the period over which the sporophores matured was virtually the same in all cases, even though the fruit-bodies were considerably larger upon the larger discs.

1) The behaviour of assimilated materials.

The distribution of the mature or nearly mature sporophores

over the surface of a circular colony appeared to be quite haphazard. This suggested that their distribution may have been governed by a nutritional mechanism, such as the following.

Some sporophore primordia commence development a little earlier than the rest. They then draw on, and deplete, intracellular reserves in the vegetative mycelium, and thereby retard or inhibit the development of other fruit-bodies. In this way, inevitable slight irregularities in the initiation of primordia become accentuated, and the normal regularity in the initial distribution of sporophores is obscured as fruiting proceeds.

According to this hypothesis, if the two halves of a culture were separated, then neither half would be able to deplete the intracellular reserves of the other, and each should then produce a similar quantity of sporophores. This would show that each half had a comparable potentiality for fruiting, and that the normal failure of intact cultures to display this equality was the result of mutual interference of their component parts.

Accordingly, two sets of five Petri dishes, each containing 20 ml. of 0.1% Alanine Agar, were inoculated. Two similar plate cultures had been established the previous day in order to indicate when fruiting was imminent in the main series of cultures.

On the 10th. day, when the then 11 days old indicator cultures first showed primordia, the following treatments were applied to the two sets.

Series (i) : a diameter of the colony was drawn across the base of each of the five Petri dishes, marking out two halves, (a) and (b).

Series (iii) : each culture was bisected with a sterile scalpel into halves (x) and (y), one half then being transferred to an empty sterile Petri dish.

All cultures were then incubated for a further 6 days. The yields of sporophores and also of vegetative mycelium from each separated half, (x) and (y), and of the hitherto unseparated halves, (a) and (b), were then determined.

In order to eliminate complications arising from the slight natural variations in total yields by these cultures, the yields of mycelium and sporophores from the two halves of each colony were expressed as percentages of the total dry weight of the colony. The mean results of this experiment are given in Table XXI, together with those of a second performed in precisely the same manner, except for being left only 5 days after halving the appropriate cultures. Mean differences in yields were derived by taking all individual differences as positive.

Table XXI.

The Effects of Fruiting upon the Distribution of Fungous Material in Cultures.

	Experiment I		Experiment II	
	Unseparated Halves	Separated Halves	Unseparated Halves	Separated Halves
Mean Yield of Vegetative Mycelium per Culture	70.3 V.M. (a + b)	59.0 V.M. (x+y)	75.8 V.M. (a+b)	72.7 V.M. (x+y)
Mean Yield of Sporophore Tissue per Culture	29.7 S.T. (a+b)	41.0 S.T. (x+y)	24.2 S.T. (a+b)	27.3 S.T. (x+y)
Mean Difference between Yields of Vegetative Mycelium from Fellow Halves.	6.3 V.M. (a-b)	6.7 V.M. (x-y)	6.3 V.M. (a-b)	3.6 V.M. (x-y)
Mean Difference between Yields of Sporophore Tissue from Fellow Halves.	23.3 S.T. (a-b)	3.6 S.T. (x-y)	24.2 S.T. (a-b)	1.6 S.T. (x-y)
Mean Difference between Total Yields of Fungus from Fellow Halves.	18.2 T.F. (a-b)	4.5 T.F. (x-y)	11.1 T.F. (a-b)	4.3 T.F. (x-y)

N.B. Units are percentages of total yields (a + b, and x + y) per culture.

All differences are taken as positive.

V.M. = vegetative mycelium; S.T. = sporophore tissue, T.F. = total fungus

It is clear that the mean differences between yields of sporophores from fellow halves were very large in the unseparated

series of cultures (see S.T. (a - b)), but between the separated halves they were very small (S.T. (x - y)).

The hypothesis is therefore substantiated by these experiments.

It was also found that although the total yields of fungus from unseparated halves differed greatly, as compared with the separated halves, the differences were almost entirely attributable to the irregular distribution of sporophore tissue, for the differences in yields of vegetative mycelium from fellow halves were of comparable smallness in both series of treatments.

II. Physical Factors.

a) Light.

1) The effects of exposure to light.

Voderberg (1949) recorded that the onset of fruiting of Coprinus lagopus, cultured upon bacterium-containing horse dung, was scarcely influenced by light. Her data showed that at 25°C. there was no effect, while at other temperatures cultures fruited one day earlier in darkness than in light.

To investigate this result, a number of cultures were established upon autoclaved horse dung in Petri dishes. Six of the cultures were maintained at 25°C. in continuous illumination at about 8 foot-candles, while the rest were wrapped in black paper and kept in light-proof tins. At the first appearance of primordia in the illuminated cultures, a sample of three

was removed, from the series in the dark, taking every precaution that no light was admitted to the rest. Observations on fruiting were made, and further samples taken at daily intervals.

It was found that one of the six illuminated cultures began fruiting on the 8th. day, and all six replicates had begun by the 11th. (mean age at onset of fruiting, 9.5 days), while none of the samples taken from the dark bore any primordia until the 16th. day, when all replicates were fruiting.

Radial growth in darkness was slightly higher than in the light, the respective mean rates over the period 8th-12th. days inclusive being 4.25 and 3.4 mm. per day.

Cultures taken from the dark were exposed for up to two hours while observations were taken. They were then returned to the light-proof tins. Subsequent observations showed that hitherto non-fruiting cultures began to produce primordia either one or two days after this exposure to light.

A comparison of vegetative and reproductive growth in light and darkness was made upon 0.1% Alanine Agar. Cultures with laterally placed inocula were kept in light or total darkness, and samples of four taken at intervals over a period of 19 days. Growth and fruiting were recorded quantitatively; the results are presented in Fig. VII.

It was clear that cultures fruited much earlier in light than in darkness. The reduction in weight of vegetative mycelium was again associated with prolific sporophore production; this confirmed results recorded in Table IX. Radial growth was

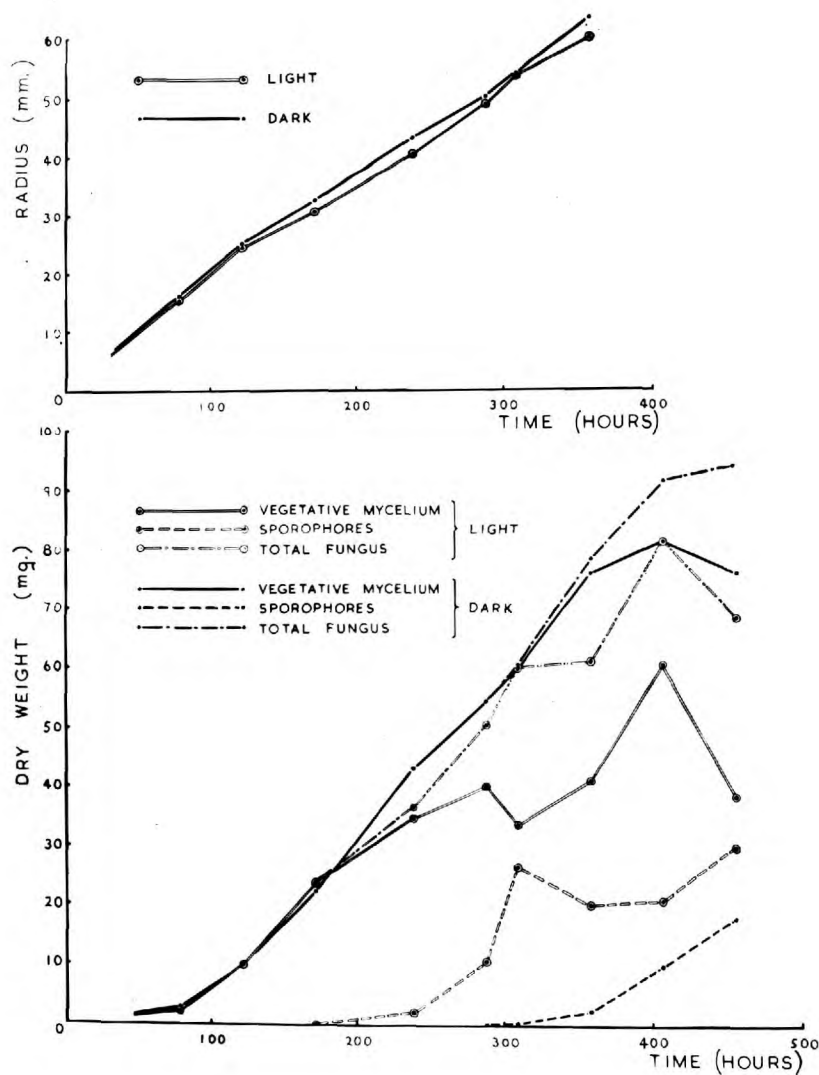


Fig. VII. The Effects of Light and Darkness on Radial Growth (above) and the Distribution of Growth in Weight on 0.1% Alanine Agar.

scarcely affected by light (Fig. VII).

In later experiments, gravimetric data on the effects of different light treatments were obtained at only one date, usually about the 16th. day of incubation. Significant differences in yield were taken as indicative of differences in the date of onset of fruiting.

ii) The quantity of light stimulating fruiting.

Preliminary experiments on various media indicated that continuous light was not necessary to stimulate fruiting, and that an exposure of only a few minutes at about the time fruiting would normally commence was sufficient. The following experiment was therefore conducted to determine the minimal exposure necessary.

Of forty cultures upon 0.1% Alanine Agar, five were kept in continuous light while the remainder were incubated in complete darkness. On the ninth day, the cultures in darkness were exposed to light from an incandescent tungsten filament bulb at an intensity of 250 foot candles* for periods ranging from 0 to 5 minutes, each treatment being in quadruplicate. These cultures were returned to darkness for a further five days. Growth and fruiting were then assessed qualitatively; the results are given in Table XXII.

* The intensity was measured with an EEL Photometer (Evans Electroselenium Ltd) at the level of the culture surface, but before reaching the culture the light would first pass through the side of the medical flat.

Table XXII.

The Effect of Different exposures to Light at 250 f.c. on Growth and Fruiting.

Duration of Light Exposure (Seconds)	Mean Dry Weight (mg.)		
	Mycelium	Sporophores †	Total
0	69.2	4.2	73.4
1	52.0	22.7	74.7
5	53.3	20.4	73.7
10	50.1	26.0	76.1
60	49.8	25.3	75.1
180	41.7	29.9	71.6
300	45.1	27.7	72.8
Continuous Exposure	33.0	24.4	57.4

† Significant difference between mean dry weights of sporophores

at { p. = 0.05, 9.9 mg.
{ p. = 0.01, 13.4 mg.

An exposure as short as one second was sufficient to accelerate fruiting; longer exposures had no further effect.

It will be noted that the yield of vegetative mycelium in continuous light was low. Similar observations were made in other experiments, and are discussed later (p.109).

In a further experiment, cultures were exposed for the same time to lights of different intensities.

One series of cultures was incubated in total darkness for ten days, and another series kept at the same temperature in continuous light. On the 10th. day, the cultures in darkness were given five seconds' exposure at light intensities ranging from 0 to 250 foot-candles, and then returned to darkness for a further five days. The various intensities were obtained by varying the

distance between the cultures and a 200 watt bulb, for intensities greater than 50 foot-candles, or a 25 watt bulb for 10 foot-candles and less.

Table XXIII

The Effects of 5-second Exposures to Lights of Various Intensities on Growth and Fruiting.

Intensity of Light Exposure. (foot-candles)	Mean Dry Weight (mg.)		
	Mycelium	Sporophores †	Total
0	76.1	8.4	84.5
0.01	71.4	14.0	85.4
0.1	59.7	19.5	79.2
0.5	65.8	19.1	84.9
2.5	58.9	21.8	80.7
10	63.1	21.3	84.4
50	62.2	19.7	81.9
250	57.0	24.5	81.5
Continuous Light	33.7	23.7	57.4

† Significant differences between mean dry weights of sporophores.

at { p. = 0.05, 7.4 mg.
p. = 0.01, 10.0 mg.

The results (Table XXIII) show that a significant increase in yield of sporophore tissue was produced by an intensity of 0.1 foot candles, and that higher intensities did not increase the yield further. Cultures exposed to light intensities of 0 or 0.1 foot-candles lacked the profusion of primordia over the surface of the colony which was to be seen in those exposed to greater intensities.

iii). The onset of sensitivity to light.

In the previous experiments, cultures were exposed to light

at, or shortly before, the time when primordia would normally have appeared in continuous light. The age at which colonies became sensitive to light was investigated with plate cultures on 0.3% Yeastrel Agar incubated in total darkness.

On each day after the third, sets of four plates were removed from darkness and exposed to unstandardized daylight for five minutes. During exposure, a ring was drawn in Indian ink on the bottom of each plate around the margin of the colony. The cultures were then returned to darkness. All plates were examined on the 12th. day. Fruiting, which at this stage consisted only of primordia, was recorded in terms of numbers of the latter, and these data are given in Table XXIV.

Table XXIV.

The Effect on Fruiting of Exposing to Light Cultures of Different Ages.

Age when Exposed (Days)	Mean Diameter when Exposed (mm.)	Mean number of Primordia.
3	-	0.25
4	22.4	0.75
5	33.1	8
6	43.5	180
7	50.6	285
8	55.6	Approx. 450
9.5	68.0	Approx. 800-1000
10	74.9	Approx. 1000-1500

Cultures exposed on and before the 5th. day had no primordia or a few very small ones. Cultures exposed on or after the 6th. day bore larger numbers of primordia, none of which lay outside of the line marking the margin of the colony at the

time of exposure. Their distribution differed somewhat according to the age at the time of exposure. Up to the 8th. day they were more or less uniformly distributed, except in the immediate vicinity of the inoculum where they were sparse. On and after the 9th. day, the main mass of primordia did not extend up to the inked line, there being a primordium-free zone immediately within it, and then a well-marked ring of primordia coinciding with what was the margin at the time of exposure. The main mass of primordia occupied an area equivalent to the growth made in eight days.

The onset of sensitivity to light was studied in a further experiment in which fruiting was assessed in terms of dry weight. 0.1% Alanine Agar in medical flats was used. Observations were made on two dates and the results given in Table XXV.

Table XXV.

The Effect on Growth and Fruiting of exposing to Light Cultures of different Ages.

(a) Incubated 14 days.

Age at time of Exposure (Days)	Mean Dry Weight (mg.)		
	Mycelium	Sporophores †	Total
3	80.2	0.2	80.4
4	75.9	0.1	76.0
5	84.7	0.1	84.8
6	83.8	0.3	84.1
7	75.9	9.1	85.0
9	63.8	18.4	82.2
11	75.7	5.2	80.9
No Exposure	81.9	1.5	83.4
Continuous Exposure	42.7	28.4	71.1

† Significant differences between mean yields of sporophores

at $\begin{cases} p. = 0.05, & 7.9 \text{ mg.} \\ p. = 0.01, & 10.7 \text{ mg.} \end{cases}$

(b) Incubated 20 days.

13	50.0	26.7	††	76.7
16	50.8	24.8		75.6
No Exposure	55.4	21.2		76.6

†† No significant differences between Means.

The sensitivity to light developed in this case on the 7th. day or slightly later. The mean yield of sporophores from cultures exposed on the 7th. day was lower than that of those exposed on the 9th., primarily because two of the four replicates had not responded to the light. The yield from cultures exposed on the 11th. day was low owing to the short interval (3 days) between initiation and harvesting. Fruiting in

cultures exposed on or after the 13th. day was not significantly greater than in those kept in darkness.

iv). The rapidity and progress of the response to light exposure.

It had been observed on various occasions during this work that the response to light exposure of cultures which were, as it were, ready for fruiting, was extremely rapid. The speed of the response was determined in the following experiments in which the behaviour of the isolate was recorded during the period between exposure and maturation, or the highest stage of sporophore development attainable in the dark.

Cultures with laterally placed inocula were established on 0.1% Alanine Agar. Five were kept in continuous light, and the remainder kept in darkness. On the 10th. day when three of the five cultures in continuous light had begun to form primordia, thirty-five of the cultures in darkness were exposed for five minutes to light at 250 foot-candles from a tungsten filament lamp. Thirty were then returned to darkness, and the other five taken as a sample. On successive days, further samples of five were removed from darkness, the final sample being taken on the 16th. day. The yields of mycelium and sporophore tissue of each sample were determined at the time of sampling. Those of cultures in continuous light and continuous darkness were determined on the 16th. day. The results are given in Table XXVI.

Table XXVI.

The Progress of Fruiting after Exposure to Light.

Age of Sampling (Days)	Mean Dry Weight (mg.)			Observations on Fruiting.
	Myc.	Sporoph.	Total	
10 (at exposure)	36.0	0	36.0	No primordia.
11 (1 day after exp)	38.4	0 (+)	38.4	All with primordia.
12 (2 " " ")	55.0	0.1	55.1	Primordia enlarging.
13 (3 " " ")	52.9	2.1	55.0	" "
14 (4 " " ")	55.8	6.1	61.9	Small etiolated sporoph.
15 (5 " " ")	67.3	7.5	74.8	Larger " sporoph.
16 (6 " " ")	53.3	25.4	78.7	"Mature" sporophores. †
16 Continuous Light	31.9	22.9	54.7	Mature sporophores.
16 Continuous Dark	83.4	0.6	84.0	Few scattered primordia.

(† "Mature" sporophores were full-sized, but mostly etiolated, fruit-bodies.)

Primordia appeared after only one day (actually 27 hours), although several days elapsed before the yield of sporophore tissue was appreciable. Most primordia that developed after one day were in a ring corresponding to the margin of the colony at the time of exposure, but all sporophores which developed further were derived from primordia lying within this ring.

Again, the total weight of fungus from the series in continuous light was considerably less than that of other treatments of the same age.

v). The effects of light treatments after the initial exposure.

In a number of fungi it is not light exposure alone which is necessary for sporulation but a proper sequence of light and darkness (Barnett and Lilly, 1950; Timnick et al., 1951).

As light affects subsequent development of the individual fruit-bodies of Coprinus lagopus (Borriss 1934a, 1934 b; Voderberg, 1950), it seemed possible that the conditions of illumination after the initial exposure might influence the yield of sporophores. The following experiment was conducted to investigate this.

Thirty Petri dish cultures on 0.1% Alanine Agar were divided into sets of five. Four sets were placed in complete darkness, while two were kept in the light. Subsequent procedure was as shown diagrammatically in Table XXVII, in which the final yields of sporophores and of vegetative mycelium are recorded.

Table XXVII.

The Effects of Light and Darkness after Initiation of Fruiting.

					Mean Dry Weight (mg.)		
10	5 min.	48	78		Myc.	Sporoph.	Total
Days	at 250 f.c.	Hrs.	Hrs.			†	
-----↓-----							
a)					52.0	27.0	79.0
b)					86.4	0.3	86.7
c)					65.6	21.0	86.6
d)					63.0	19.9	82.9
e)					66.8	17.5	84.3
f)					54.6	32.2	86.8

† Significant differences between means of sporophore yields

at { p. = 0.05, 7.7 mg.
p. = 0.01, 10.5 mg.

The data clearly fall into three groups; treatments (a) and (f) with the highest yields of sporophore tissue; (c), (d) and (e) with somewhat lower yields; and (b) with practically no sporophore tissue. The sporophore yields after a particular light stimulus (either initially continuous light or a brief exposure) were virtually equal, irrespective of the subsequent treatment. It will be seen from Plate IV that the distribution of the sporophore material was quite different in these three groups.

The presence of prominent patches of vigorously growing mycelium which overgrew the surrounding colony will be noticed in the treatment in continuous darkness (Plate IV, b). These

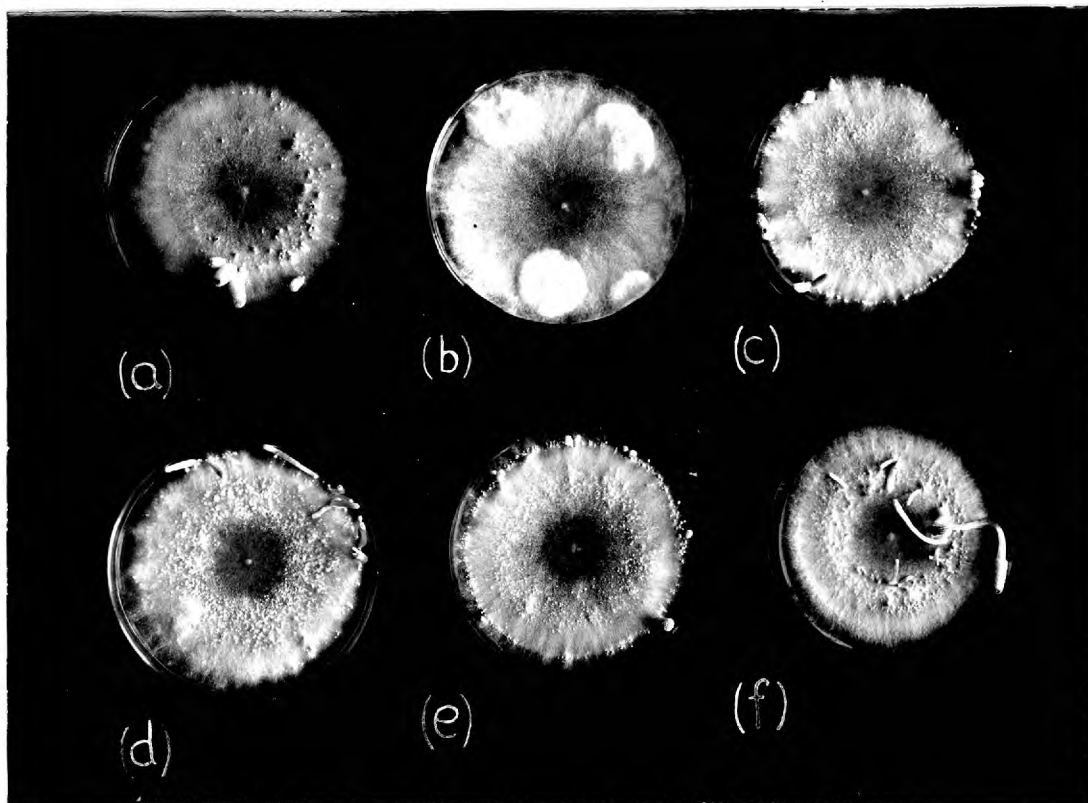


Plate IV. The effects of light treatments, after an initial exposure, on colonies of Coprinus lagopus.

(See Table XXVII for key to treatments).

appeared very commonly under such conditions, but were not seen in cultures grown in continuous light.

vi). The effect of light of different wavelengths.

The work of Borriss (1934 b) showed that the sporophores of Coprinus lagopus were very sensitive to the wavelength of light acting as a tropic stimulus. The maximal sensitivity was to light of wavelengths between 4,400 and 4,500 Å (dark blue); light of wavelengths greater than 5,900 Å was practically ineffective. The sensitivity to blue light was about 1,000 times greater than to green. Similarly, as a formative stimulus affecting sporophore development, light of wavelengths between 4,000 and 5,000 Å was very active, while that of wavelengths over 6,400 Å evoked no response.

It was of interest to see if wavelength was important in light acting as an accelerator of fruiting.

A number of cultures upon 0.1% Alanine Agar was therefore exposed, after 9 days of incubation in the dark, to lights of various colours. Control cultures were maintained in continuous white light.

The exposures to coloured lights were standardized so that all cultures received similar quantities of radiant energy at their surfaces. This was done with an EEL Photometer and a spectral response curve appropriate to the instrument. Absorption by the glass wall of the culture vessel was disregarded. The intensities of light, as read by the photometer, were all adjusted to values equivalent to 2.5 foot-candles for light through a filter with maximal transmission at 4,500 Å (blue).

The colour filters used in this experiment had a fairly wide band transmission, and are listed in the table of results by the wavelength at which their transmission was maximal.

Exposures of five seconds and five minutes were used at each wavelength except the shortest, when five seconds' exposure alone was given. The exposures were given at 25°C., the temperature of incubation during the whole experiment. After exposure and a further five days' incubation, the response was measured gravimetrically (Table XXVIII).

Table XXVIII.

The Effects of Exposure to Lights of different Colours on Growth and Fruiting.

Light Exposure.		Mean Dry Weight (mg.)		
		Mycelium	Sporophores [†]	Total
Red (6600Å)	5 seconds	71.6	1.0	72.6
	5 minutes	67.2	2.4	69.6
Orange (6000 Å)	5 seconds	74.4	1.0	75.4
	5 minutes	73.2	2.2	75.5
Yellow (5700Å)	5 seconds	67.5	3.1	70.6
	5 minutes	69.3	2.9	72.2
Green (5200Å)	5 seconds	70.8	2.3	73.1
	5 minutes	64.2	12.0	76.2
Blue-Green (4950Å)	5 seconds	58.7	14.0	72.7
	5 minutes	61.2	13.7	74.9
Blue (4500Å)	5 seconds	56.1	15.5	71.6
White.	Continuous	33.1	24.2	57.4

[†] Significant differences between mean dry weights of sporophores

at { p. = 0.05, 3.9 mg.
p. = 0.01, 5.2 mg.



Plate V. The effects on Coprinus lagopus of exposure to lights of different colours.

It is clear that fruiting was stimulated only with the shorter wavelengths, green (5,200 Å) occupying a transitional position, in that fruiting was hastened with a five minute exposure, but not with five seconds. In those treatments in which the wavelength exceeded 5,200 Å, fruiting consisted of small etiolated sporophores (Plate V).

vii) The distribution of the response to light.

In a previous experiment in which the development of sensitivity to light was investigated, it was noted that in no case did primordia develop upon mycelium formed after the exposure to light. The implications of this result were that either the stimulus was not translocated, or was translocated, but only in a centripetal direction, or was translocated in all directions, but tissues formed after exposure were incapable of response.

The discovery that the fungus was insensitive to red light provided a means whereby the colonies could be manipulated in conditions which did not affect fruiting. Use was made of this in the following experiment in which it was investigated whether or not the stimulus was transmissible.

Ten Petri dish cultures upon 0.1% Alanine Agar were incubated in total darkness for ten days, and then, in red light, one disc 17.5 mm. in diameter was cut with a sterile cork-borer half way along a radius of each colony, and transferred aseptically to an empty sterile Petri dish. Five of the discs were then returned immediately without further treatment to the cultures from which they were taken. The remaining five were exposed to daylight, at approximately 50 foot-candles, for 15 minutes before they too

were restored to the appropriate cultures. The cultures were returned to the 25°C. incubator for a further 5 days, after which all were examined, and the yields of vegetative mycelium and sporophores determined separately for the discs and the rest of the colonies.

Table XXIX.

The Effect of Localised Light Exposure on Distribution of Fruit-Bodies.

Treatment	Disc. Dry Weights(mg)			Rest of Culture Dry Weights (mg.)			Total Fungus (mg.)
	Myc.	Sporoph.	Total	Myc.	Sporoph.	Total	
Disc Exposed	3.0	15.0	18.0	67.2	0.3	67.5	85.5
Disc not Exposed.	3.7	0	3.7	79.5	2.3	81.8	85.5

The results given in Table XXIX clearly show that the fruiting after exposure was confined to the exposed discs, on each of which a clump of sporophores had developed. On the rest of the colony, three of the five replicates had one to a few very small primordia of negligible weight. It was evident that organic continuity between the discs and the rest of the mycelium was re-established because the total weight of fungal tissue on the exposed discs was practically five times that upon the unexposed, this being associated with a comparable reduction in total weight of the rest of the colony, from which, presumably, materials were transferred.

A similar experiment was conducted upon a further ten cultures, removing a pair of discs from each culture from

opposite radii. The results were essentially similar to those presented above.

In order to further confirm these results, seven plate cultures grown in darkness for ten days were halved aseptically, and whole halves were exposed to day light at 50 foot-candles for five minutes before returning to their parent cultures. Another series of seven cultures was similarly treated, except that the removed halves were not exposed to white light.

Table XXX.

The Effects of Exposing only One Half of a Colony to Light.

Treatment	Unexposed (or undisturbed) Half. Dry Weights. (mg.)			Exposed (or disturbed) Half. Dry Weights. (mg.)			Total Fungus (mg.)
	Myc.	Sporoph.	Total	Myc.	Sporoph.	Total	
One half exposed, other unexposed.	27.9	0	27.9	19.2	37.2	56.4	84.3
Neither half exposed.	44.5	3.1	47.6	43.0	0.7	43.7	91.3

The results after a further six days' incubation in darkness show again that, except for single primordia of negligible mass which appeared in each of two replicates, fruiting was confined to those halves of the cultures exposed to the light. In the unexposed controls, fruiting was sparse. It will be noticed that the mean yield of vegetative mycelium from the non-fruiting halves of the treated colonies was almost as low as that of the fruiting halves, and certainly much lower than that in the controls where both series of halves agreed closely.

There was however comparatively little difference in the total weight of cultures from both treatments. This indicated that fruiting on the exposed halves of treated cultures involved translocation of materials from the unexposed halves.

b) Temperature.

According to Voderberg (1949), the optimal temperature for mycelial growth and fruiting of Coprinus lagopus is between 20 and 25°C. At temperatures above 30 and below 10°C., no fruit-bodies developed, although mycelial growth continued weakly. At temperatures from 10 to 20°C., the appearance of the first fruit-body was delayed one to three weeks. Similar results were obtained in both light and dark.

In the following experiment, the temperature response was studied over a period of fifteen days. Four cultures on 0.1% Alanine Agar were incubated at each of several temperatures from 10 up to 39°C., and were exposed to daylight for at least several minutes each day. Previous work had shown that light exposures as short as this were as effective stimuli of fruiting as continuous illumination. The response was recorded gravimetrically (Table XXX).

Table XXXI.

The Effect of Temperature on Growth and Fruiting.

Mean Dry Weights (mg.)	Incubation Temperatures ($^{\circ}\text{C}.$)						
	10	15-19	20	25	30	35	39
Mycelium	17.2	61.0	66.6	45.2	45.6	1.3	0
Sporophores	0	0(+)	0.6	27.6	0	0	0
Total	17.2	61.0	67.2	72.8	45.6	1.3	0

(+) Indicates fruiting, but of negligible yield.

This experiment was repeated with the cultures kept in complete darkness for the same period, viz. 15 days. The results are shown in Table XXXII and Plate VI.

Table XXXII.

The Effect of Temperature on Growth and Fruiting in Darkness.

Mean Dry Weights, (mg.)	Incubation Temperature ($^{\circ}\text{C}.$)						
	10	15-19	20	25	30	35	39
Mycelium	12.9	58.9	68.2	77.9	20.9	6.7	0
Sporophores	0	0	0(+)	10.4	0	0	0
Total	12.9	58.9	68.2	88.3	20.9	6.7	0

(+) indicates fruiting, but of negligible yield.

The general behaviour of the cultures was similar in both experiments. The optimal temperature for both total growth and fruiting being $25^{\circ}\text{C}.$ There was virtually no fruiting at any other temperature tested and, as earlier experiments had



Plate VI. The Effects of temperature on growth and fruiting of Coprinus lagopus in darkness.

shown, fruiting as recorded on the 15th. day was considerably reduced by total darkness at 25°C.

It has been reported for a number of fungi that dependence of fruiting on light can be offset by incubation at a sufficiently high temperature. Thus Leonian (1924) recorded that although Sphaerographium fraxini was unable to produce pycnidia in the dark at room temperatures, a few were produced at 30°C.

It therefore seemed possible that the partial dependence of Coprinus lagopus on light for fruiting at 25°C. might be a total dependence if the temperature were sufficiently low. In the two previous experiments with relatively short incubation periods fruiting had barely commenced at temperatures under 20°C., and there was none at 10°C. Accordingly, in the following experiment, cultures were incubated at 10, 15-19, 20 or 25°C. in both intermittent light and continuous darkness for 29 days. During this period daily observations were made on the series exposed to light. The only observations on the dark series were made on the final day.

Table XXXIII.

The Effects of Temperature upon Fruiting in Light and Dark.

Temperature °C.	Intermittent Light		Continuous Darkness
	Age at Onset of Fruiting (Days).	Age at Maturation (Days).	Observations on Fruit- ing on 29th. day
10	23	> 29 (Sporoph. all less than 1 cm. high.)	All replicates with small primordia near sides of medical flat.
15 - 19	13	24	Sporophores large, mostly etiolated.
20	10	19	"
25	8	13	"

It will be seen from Table XXXIII that at none of the temperatures used was fruiting prevented in darkness. The onset of fruiting was increasingly delayed as the temperature was lowered, and the period required for maturation of the sporophores increased, until at 10°C. this was not completed at the end of the experiment.

c) Aeration.

The effect of reduced aeration was studied in cultures growing on 20 ml. of 0.1% Alanine Agar in each of sixteen 8 oz. medical flats. This size, which was smaller than that used in other experiments, was adopted so that possible effects of restricted gas exchange would be accentuated. Eight of the vessels were loosely plugged with cotton wool, and the remainder

sealed with gas-tight screw caps. Observations on radial growth and fruiting were made periodically, and, in samples of four taken upon the 12th. and 19th. days, the yields of vegetative mycelium and fruit-bodies were also determined (Table XXXIV).

Table XXXIV.

The Effect of Aeration upon Growth and Fruiting.

	Aerated	Non-aerated
Mean Diameter on 10th Day, (mm.)	84	65
" " " 12th " "	106	67
" " " 19th " "	>130	67
Mean Days to begin Fruiting.	8.5	9.1
Mean Dry Weights { Mycelium on 12th Day, { Sporophores (mg.) { Total	39.7 33.4 73.1	47.3 7.5 54.8
Mean Dry Weights { Mycelium on 19th Day, { Sporophores (mg.) { Total	22.8 34.8 57.6	25.6 2.6 28.2

At the first sampling, the aerated cultures all bore mature or nearly mature sporophores. By the 19th. day these were deliquescing and a second crop had matured. The non-aerated cultures had by the 12th. day a fair number of primordia and some sporophores at an early stage. By the 19th. day, these had developed into abnormal fruit-bodies not exceeding 8 mm. in height. Each had a relatively large stipe-base, abruptly narrowing to a slender, short stipe bearing a rudimentary pileus.

The data also show that the non-aerated cultures lost weight over the period between the sampling dates in spite of the poor production of sporophores, none of which had deliquesced. Although radial growth virtually stopped after the 10th. day, primordia appeared almost as soon in these as in the aerated cultures.

D. DISCUSSION

The isolate of Coprinus lagopus used in this work was able to utilize cellulose, as was that used by Voderberg (1948), but it is clear that simpler carbohydrates could also be consumed, and were able to support well developed crops of fruit-bodies. This result was rather more expected than that of Voderberg which led to her conclusion that the organism, through becoming so closely adapted to its natural substrate, was no longer able to use carbohydrates other than cellulose, insofar as one must believe that insoluble polysaccharides were enzymically hydrolysed to simpler soluble carbohydrates before they could enter the cells of the fungus. Such an hydrolysis was demonstrated in the case of starch.

The apparent need for cellulose was possibly caused by contaminating substances, e.g. growth factors, present in the materials which Voderberg used, viz., finely ground straw and blotting paper. The presence of such factors has been demonstrated in cotton wool (Robbins and Ma, 1942) and in jute (Basu, 1947), so it is not unlikely that they are present in the sources mentioned above.

Voderberg (1948) found the presence of sterilized yeast was also necessary for good growth, so that the isolate of C.lagopus that was used was probably deficient for a number of factors. Since the isolate used in this work developed a further deficiency which could largely be satisfied by an extract of agar, and C.lagopus is known to have many sexual strains

(Hanna, 1925), it is not impossible that different isolates differ in their nutritional requirements.

It is interesting to note a certain specificity with regard to the carbon source. Thus while sucrose was not used, glucose and fructose were readily utilized (Table I). It was noted that adjacent to a bacterial contaminant which appeared in a culture upon a sucrose medium the otherwise very thin mycelium became quite dense, no doubt as a result of the conversion of the sucrose into usable monosaccharides by invertase secreted by the bacteria.

The cellulolytic properties of the isolate are understandable since cellulose is a major constituent of its natural substrate, horse dung. The ability to hydrolyse starch is less readily appreciated, for it is unlikely to be present in horse dung in large quantities. Iodine tests failed to demonstrate its presence.

The inhibitory effects of galactose and lactose are not easily explained. C.lagopus appeared to lack enzymes hydrolysing lactose, for it has been shown that the products of this hydrolysis, viz. glucose and galactose, together supported good growth and gave a characteristic growth form to the colony. Thus lactose must in itself have been inhibitory. In contrast, raffinose, again not hydrolysed by this organism, had no inhibitory effect on growth.

Having accepted that lactose could restrict the growth of this fungus, there is little difficulty in interpreting its effect in the presence of glucose. However, galactose in

these circumstances actually led to the more effective utilization of glucose, or itself became usable, for growth, although erratic, was nevertheless on average far greater than with either sugar alone. More work would be necessary to analyse these effects further.

Zonation in fungi is not an uncommon phenomenon, and is usually related to some rhythmical change in the environment, such as fluctuating temperature or alternating light and dark (Hedgcock, 1906; Bisby, 1925; Hall, 1933). However, in a number of cases zonation has been reported in uniform external conditions (Hedgcock, 1906; Hein, 1930, a). The zoning of C.lagopus on glucose-galactose medium was of this type.

Hein (1930 a), describing this sort of zonation in an unnamed sclerotium-forming fungus, suggested that a process took place within the agar substrate similar to that which produces the inorganic phenomenon known as Liesegang's rings, and that the rings so formed provided specific conditions which initiated either fruiting or the dense aggregation of hyphae of the fungus. A point of difference with the example described by Hein is that whereas he set forth the factors which initiated and controlled sclerotium-production as beginning abruptly, to diminish gradually as the ring widened, in C.lagopus the converse was true, unless the reaction was considered in terms of rate of radial growth rather than density of mycelium and primordium production (see Fig. I and Plate II). A closer comparison of this result with that of Hein is not possible since the media he used were prepared from natural materials

(prune agar and lima bean agar). It is however interesting to note that the zonation he described did depend on the substrate, being restricted to certain media.

The reaction of C.lagopus to initial concentration of carbohydrate was similar to that of Melanospora destruens (Hawker, 1939), insofar as fruiting increased to a maximum and then declined as the initial glucose concentration was raised, while mycelial growth continued to increase to a maximum at a higher level of glucose, before it too diminished. The response can perhaps be more closely compared with that of Collybia velutipes (Plunkett, 1953). In this fungus the maximal yield of sporophores was attained at a lower initial carbohydrate (sucrose) concentration than that optimal for mycelial growth. The effect on fruiting of further increases in carbohydrate concentration was to cause an increasing number of replicates to fail to fruit, while the yield of those which did differed but little from the maximum. This was not wholly the case in C.lagopus, for although the number of fruiting cultures declined with supra-optimal increments of glucose, the actual yield of sporophores by individual fruiting cultures became restricted through the sporophores failing to develop fully. So greatly was yield reduced above 1% glucose (Table IV) that it is doubtful whether the primordia produced at 2% and 3% glucose would ever have matured, for their rate of development, so far as it was observed, was very slow. This diminution of yield could therefore be only in small part attributed to the delay in appearance of primordia which was brought about by high glucose concentration.

It is probable that the apparent antagonism sometimes seen between vegetative and reproductive growth (p.22) was the result of the different optima of these two phases of growth for concentration of carbohydrates. The reaction of a culture to an increased level of carbohydrate would depend on the nutrient status of the medium, relative to these optima, prior to making the addition.

Although the acid drift in the medium increased with increased initial concentration of glucose, it is doubtful whether the magnitude of this - at most little more than one pH unit below the initial value of 8.0 - was sufficient to account for the adverse effects on fruiting of high levels of glucose.

That the concentration of glucose in the medium was not reduced to a uniform low level at the time fruiting commenced has already been described. However it should be borne in mind that when fruiting entails the production of so large and complex a structure as a sporophore, the prerequisite conditions for each stage of development may be very different. On morphological grounds alone, five stages of development of fruit-bodies of Coprinus lagopus have been recognized (Borriss, 1934 a) and it can readily be shown that at various stages the sporophore becomes particularly sensitive to one or other environmental factor, such as light or gravity. Plunkett (1953) recognized several stages of development of the fruit-bodies of Collybia velutipes, each governed by particular factors

of the environment. Accordingly it may well be that the conditions in the substrate for laying down sporophore primordia are not in themselves suitable for their further enlargement and elaboration. Thus although the formation of primordia may not be strictly controlled by glucose concentration, although undoubtedly affected by it, later development may be more intimately governed by this factor. The retarded processes of sporophore development in media with high glucose point this way, but further work is necessary before the measure of this control can be determined.

The range of nitrogen sources utilized covered both organic and ammonium-nitrogen, but not nitrate-nitrogen. This result was rather intermediate between that of Leonian and Lilly (1938) who found only organic nitrogen could be used, and Voderberg (1948) who recorded that all sorts of nitrogen were usable, although organic sources were better. These differences are not irreconcilable. Leonian and Lilly conducted their series of experiments in media adjusted to pH 5.5. Results presented earlier show that the presence of calcium carbonate greatly enhanced the usability of ammonium-nitrogen, presumably by maintaining a favourable pH against the tendency for acid drift. If then the pH drift in unbuffered media, initially at about pH 7.3, was sufficient to reduce the use of ammonium-nitrogen, the relatively low pH of 5.5 might virtually inhibit it.

In the case of Voderberg's result, the basal medium she used contained finely ground straw and a little sterilized

yest. This medium alone was capable of supporting some fruiting and, presumably, vegetative growth, without additional nitrogenous material. It is therefore doubtful whether the increase in fruiting obtained with potassium nitrate (6.4 to 7.2 on an empirical scale) really indicated its utilization by the organism, especially since the extent of fruiting is not always a reflection of the extent of total growth.

Of ammonium- and organic-nitrogen sources, certain of the latter were the sources most readily used without special buffering. It therefore seems likely that it is organic nitrogenous material, probably of the peptone-type, which is used in nature, as was suggested by Voderberg (1948).

The effects of varied concentration of alanine have already been discussed in part, and the effect on fruiting resolved in terms of influence upon date of appearance of sporophore primordia and influence upon individual development (p. 42).

Plunkett (1953) recorded that the total sporophore yield of Collybia velutipes was reduced, and individual development retarded by supra-optimal concentrations of asparagine, although ultimately some fruit-bodies matured at all concentrations; there was no effect of varied asparagine concentration upon the advent of the first primordia. C.lagopus however produced primordia earlier upon alanine-rich media. This effect might be correlated with the greater metabolic rate associated with increased alanine concentration, for it was shown that glucose was consumed more rapidly with high alanine than with low; in addition it might have been because of the earlier depletion of

glucose in the medium. Although this was not critical, it was at least conducive to earlier fruiting.

The peculiar response of the yield of vegetative mycelium to varied alanine concentration is clearly explicable in terms of the transfer of materials from the vegetative mycelium into the developing fruit-bodies.

It is interesting to compare the way in which varied glucose and alanine concentrations acted on vegetative growth. Increments of glucose all reduced the rate of radial growth, but, between 0.25 and 6%, had no very obvious effects on the density of mycelium. Increases in alanine concentration, on the other hand, had comparatively little effect upon radial growth but increased the density of the colony.

The interaction of glucose and alanine concentrations was not unlike the carbohydrate-nitrogen interaction described by Garrett (1953) in connexion with the initiation and subsequent development of rhizomorphs of Armillaria mellea.

The increase in glucose consumption when alanine concentration was raised may in part account for the production of sporophores, when alanine concentration was sufficiently high, at what were otherwise inhibitory levels of glucose, and also account for the early fruiting under these circumstances (Table X).

The growth factor requirements of C.lagopus on alanine medium were no greater for fruiting than those described by Leonian and Lilly (1938) for vegetative growth, i.e. a need for

an exogenous supply of aneurin alone. The quantity which need be present for fruiting was however greater than that necessary for mycelial growth. In this respect, and also in that its radial growth upon agar was not greatly affected by concentration of aneurin, C.lagopus resembles Ceratostomella fimbriata (Barrett and Lilly, 1947).

The effects of agar have been studied in connection with the availability of inorganic nitrogen sources. The fact emerged that although ammonium nitrate supported growth in a wholly chemically defined medium, its availability was greatly increased by the addition of unwashed agar or an extract of this.

That agar enabled a number of fungi to utilize ammonium nitrate was recorded by Leonian and Lilly (1938).

With C.lagopus, the stimulation was attributable to a contaminating substance and not to the agar itself, for washed agar had reduced beneficial qualities. The stimulation of total growth was much less marked with an organic source of nitrogen, viz. dl- α -alanine.

In solid media prepared from washed agar, and in basal liquid media, growth ultimately approached more or less closely that on media containing the contaminating stimulant, or in alanine medium, even exceeded it. On solid media, fruiting also reached a comparable level, but in basal liquid media, after the change in vigour described earlier, the level of fruiting showed little sign of approaching that with agar.

The effect of agar in leading to early and good fruiting

appeared, then, to be partly attributable to the physical nature of the solidified medium, and partly to the presence of this contaminating substance, insofar as interference with either had a particular adverse effect.

The active substance was probably organic, since ashed agar and the trace elements tested were without action, or with considerably inferior stimulatory effect. None of the organic acids tested had a comparable action to the agar extract.

0.1% Alanine Liquid Medium always supported much greater total growth than did Ammonium Nitrate Liquid Medium. Snell and other workers (see Snell, 1952) showed that Streptococcus faecalis had a specific need for d-alanine, in the absence of which vitamin B₆ became essential for growth, enabling the organism to synthesize it. With C.lagopus, pyridoxine, which has vitamin B₆ activity, had no effect on growth in dl- α -alanine medium. On the other hand, ammonium nitrate medium was greatly improved by the addition of agar extract, proportionally far more than was alanine medium, and agar has been shown to contain pyridoxine (Robbins and Ma, 1942). It must be added, however, that 0.02% yeast extract, which probably contained pyridoxine, although in unknown amount, stimulated growth on ammonium nitrate medium no more than did aneurin alone. Further investigations of vitamin deficiencies of C.lagopus on these two media might lead to an understanding of the stimulatory action of agar extract.

Although it has long been held that the production of fruit bodies drains materials from a large mass of mycelium (e.g.

Buller, 1931, p. 152 et seq.), there seems to be no account in the literature of this being demonstrated experimentally by direct methods. That such a transfer of materials took place was suggested by the results of experiments in which the concentration of alanine was varied (Fig. IV), and subsequently confirmed directly (Table IX, Fig. VII).

The sparser formation of aerial mycelium after fruiting commenced (Plate III) was presumably correlated with the depletion of materials within the colony by fruit-body formation.

It is interesting to note that total accretion of fungal material apparently virtually ceased during the period of organization of the fruit-bodies, and then was resumed afterwards (see Table IX, and Fig. VII).

Although in Fig. VII this effect was at least in part accounted for by loss through visible autolysis of sporophores, this was not the case in Table IX. In a number of other cases, attention has been drawn to the low total weight of cultures grown in continuous light as compared with cultures of the same age grown in darkness (Tables XXII, XXIII, XXVI, XXVIII). This difference, too, was not always explicable in terms of visible deliquescence of sporophores. It seemed to be correlated with the illuminated cultures having maintained fruit-bodies for a longer period than those in darkness. In other experiments, also, fruiting was accompanied by somewhat low total weights (Tables VIII and XXX).

These observations suggest that there was probably rapid dissimilation of respirable materials during fruiting, and that this tended to counteract the current new growth of the colony.

It would be necessary to actually measure the respiratory rate during fruiting to test this explanation.

Such an hypothesis might account for the unexpected result that varied aneurin concentration had no significant effect on total yield of fungus (Table XIII).

The finding that C.lagopus fruited on all of the quantities of medium which were tested, even as little as 0.14 ml. of Alanine Agar ($\approx$ 7.5 mm. disc), is not necessarily at variance with Buller's view that sufficient vegetative mycelium to give rise to a fruit-body of a certain minimal size is a prerequisite of fruiting in the larger fungi (Buller, 1931). It was shown that there was great variation in the size of individual mature sporophores of C.lagopus, and that the minimal size must be very small indeed (less than approximately 0.2 mg. dry weight).

Since there was such an enormous variation in size of the fruit-bodies, it was unlikely that initiation of fruiting could be governed by a nutritional mechanism alone. Whereas on very small quantities of medium, 0.3 mg. of fungus could give rise to mature sporophores, on 20 ml. of medium, far more than 0.3 mg. of mycelium was formed in the course of two or three days, yet usually about ten days elapsed before primordia appeared. Thus there was a period of vegetative growth during which time either specific fruiting substances were formed, or conditions in the medium were rendered favourable, or both. The slightly earlier fruiting on smaller volumes of medium suggest that conditions in the medium played at least some part in determining the onset of fruiting.

Although it appears that the presence of adequate intracellular reserves was not in itself the factor governing initiation, it did affect subsequent development. The depletion of these reserves by developing sporophores affected the yield of sporophore tissue by the rest of the colony. The fact that in mushroom beds, few of the first formed primordia actually mature (Levine, 1922; Hein 1930 b) is probably attributable to a similar mechanism.

It is not clear, however, why in C.lagopus all of the primordia over the surface of a colony should not have developed into mature sporophores, taking advantage of what reserves were available, unless there was some other mechanism operating to prevent this.

The isolate of C.lagopus used here differed from that of Voderberg (1949) in that the initiation of fruiting was affected by light, unless the apparent difference in behaviour was attributable to experimental technique. Unless a sampling process is adopted, the exposure when making observations would be sufficient to stimulate initiation, owing to the extreme sensitivity of the organism to light.

The dependence of fruit-body initiation upon light was only partial. If light was withheld, the processes which were otherwise hastened by exposure still ultimately occurred, and fruiting commenced. This partial dependence did not become total at any temperatures between 10 and 39°C.

The response of C.lagopus to light in this respect was different from that of Collybia velutipes (Plunkett, 1953).

The latter fungus was sensitive to the duration of the light exposure which it received each day, the yield of sporophores increasing to a maximum as the daily period of illumination was lengthened. In Fusarium discolor sulphureum there was a comparable relationship between duration of exposure and abundance of conidia (Bisby, 1925). However, in C.lagopus the light effect was apparently of the "trigger" type, the yield of fruit-bodies being unaffected by the duration of exposure or the intensity of light, provided these were above a certain minimum, or by any subsequent sequence of light and dark.

The extreme sensitivity of C.lagopus to light is shown in other connections, for instance, half a minute exposure per day being sufficient as a formative stimulus (Stiefel, 1952). The sensitivity to colour of light as a fruiting stimulus closely paralleled that in connection with formative processes and phototropism in this organism (Borriss, 1934 b). Insensitivity to the longer wavelengths so that their effects are similar to that of darkness is not uncommon in fungi (Brefeld, 1877, 1889; Purvis and Warwick, 1907; Hedgcock, 1906).

The light perceptive apparatus in C.lagopus was either not developed until the culture was about six or seven days old, or alternatively the stimulus of exposure before this age could not be stored until such a time as the fungus was capable of response. Normally, as has been shown, the response became visible within 27 hours of stimulation.

The stimulus was clearly not translocated to parts of the colony not exposed to light. Because fruiting in certain

experiments was confined to single small discs of mycelium in the midst of colonies, and materials were concentrated in those discs as a result of fruiting (Table XXVIII), it is more probable that light exposure led to the production of a stimulant of fruiting, rather than to the removal of an inhibitor, for it is likely that were the latter the case, the influx of intracellular materials would have introduced more inhibitor into the discs from elsewhere.

The effects of the age at which fruiting, by appropriate exposure to light, is permitted to commence, upon the distribution of primordia and mature sporophores (Plate V) is an aspect which would repay further investigation. The limited data at hand suggest that the distribution was governed to an extent by conditions in the substrate, and that primordia could be produced under conditions which were not necessarily favourable for their further development.

Bad aeration led ultimately to the growth of mycelium and of fruit-bodies becoming virtually inhibited, the shape of the sporophores suggesting the progressive deterioration of conditions. The actual loss in weight as the cultures aged was undoubtedly the consequence of anaerobic respiration.

Relating these results to Klebs' views on fruiting in fungi, it is clear that in general they conformed to the principles which he set out, except with regard to whether or not conditions favouring reproduction were always more or less unfavourable for vegetative growth. Since fruiting removed intracellular

materials from the vegetative mycelium, it could not be determined by measurements of weight whether vegetative growth at fruiting became reduced. Using radial growth as an index, it is shown by Fig. IV that the onset of fruiting did not necessarily coincide with a reduction in growth rate.

It has also been shown (Fig. VII) that if fruiting was prevented simply by withholding light, growth in terms of radial extension and increase in weight continued at a uniform rate even though the conditions in the medium were favourable for fruiting, as was shown by the sporulation of the parallel series of cultures exposed to light.

On the other hand, the glucose concentration which was optimal for fruiting was clearly not the most favourable for vegetative growth.

Plunkett (1953), in respect of the response of Collybia velutipes to sucrose concentration, questioned the consistency with which this Hymenomycete also conformed to this principle of Klebs.

E. SUMMARY

1. Of those tested, the best sources of carbon for growth of C. lagopus were the carbohydrates, viz., glucose, fructose, maltose, xylose, cellulose, and starch. Some carbohydrates were not used, e.g. sucrose and raffinose, while galactose and lactose were inhibitory. Growth with non-carbohydrate carbon sources was poor.
2. Galactose in the presence of 0.5% glucose stimulated and led to zonation of vegetative growth.
3. The optimal glucose concentration for vegetative growth was higher than that for fruiting. Concentrations above the optimum for fruiting delayed or prevented sporophore initiation. Neither the concentration of reducing sugar nor the quantity of vegetative mycelium in particular media at the onset of fruiting were critical.
4. Ammonium salts and certain organic nitrogen sources were utilized; nitrate-nitrogen was not used.
5. Alanine concentration affected yield of mycelium in a manner explicable in terms of an experimentally demonstrated transfer of material from the vegetative mycelium into developing sporophores. Increases in alanine concentration increased glucose consumption and hastened fruiting, but above an optimum had adverse effects on

- individual development and total yield of sporophores.
6. Increases in either glucose or alanine concentration tended to counteract the adverse effects of supra-optimal concentrations of the other.
 7. Aneurin was essential for growth and fruiting.
 8. Of several organic acids tested, only acetic acid improved growth.
 9. Agar stimulated growth and fruiting. An extractable contaminating substance, probably organic, was mostly responsible for this action, although the solidification with agar itself affected fruiting.
 10. Sporophores matured on all volumes of media tested. Increased volume delayed fruiting and led to proportional increases in yields of vegetative mycelium, sporophore tissue and to some extent of sporophore size.
 11. Fruiting was hastened by very small exposures to light of an appropriate colour. This sensitivity first appeared in cultures 6-7 days old. Fruiting in response to light exposure was restricted to exposed parts of the colony.
 12. The yield of fruit-bodies was unaffected by excess light exposure or subsequent light treatments.
 13. Growth and fruiting were optimal at 25°C.
 14. Poor aeration led to arrested growth and abnormal fruiting.

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