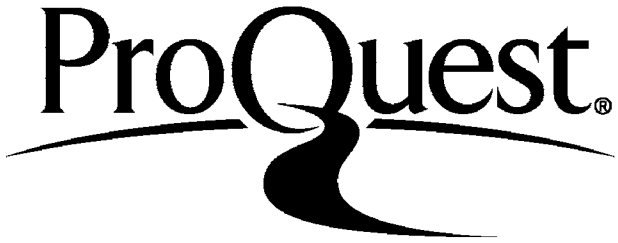
ON THE GROWTH OF BOLETUS ELEGANS SCHUM.

IN PURE CULTURE.



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THESIS SUBMITTED FOR M.Sc. DEGREE BY

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ABSTRACT

The thesis consists of two parts; the first is concerned with the morphological characters of Boletus elegans in pure culture and contains a description of the mycelium of the fungus which is compared with the descriptions of the mycelia of other Boletus spp. given by previous workers. The second and larger part deals with certain aspects of the physiology of B. elegans. Chief consideration is given to the effect of the pH value of the medium on the growth of the fungus. The pH range is found to differ significantly on ~~the~~ silica gels and gelatin media and an attempt is made to elucidate the phenomena shown in the gelatin media. In connection with the investigation into the relation between ~~the~~ pH value and growth, attention is drawn to the influence of staling products present in the inocula on the rate of growth of the cultures derived from them and to the effect upon growth of the molecular concentration of the medium. A brief enquiry into the nitrogen diet of B. elegans is also included.

INTRODUCTION

For many years considerable interest in the Hymenomycetes of woodland soils has centred round their possible rôle as mycorrhiza formers. A number of investigations have been directed to determining which species are responsible for mycorrhiza development in certain trees, either by means of observations in the field, or by synthesis experiments under controlled conditions.

Among the Boleti, B. elegans has attracted some attention largely because it is rarely, if ever, found fruiting except in the vicinity of Larch trees, a fact which immediately suggests the existence of a close relationship between the two organisms.

Melin (1922) published an enquiry into the mycorrhizal relationships of Larix decidua. He conducted synthesis experiments with B. elegans and seedlings of L. decidua and obtained evidence of mycorrhiza formation; he formed the opinion that B. elegans was an obligate Larch fungus. Melin did not however investigate the physiological properties of B. elegans in pure culture in any detail, and he gave only a brief description of the mycelial characters. In 1923 Melin referred to certain features of the mycelium of B. elegans when describing the mycelia of other Boletus spp, and in a later work (1925) he

referred to certain physiological reactions of the fungus in pure culture in connection with an enquiry into the physiological characteristics of various mycorrhiza-formers. Discussion of the individual points raised by Melin in these papers has been reserved for the appropriate places in the present paper. One concludes from Melin's work that B. elegans is of interest as a very specialised member of its genus, differing from other species in certain important mycelial characteristics such as branching, and in common with other Boletus spp. showing marked differences in certain physiological features from wood-destroying Hymenomycetes.

In 1923 Hammerlund showed that B. elegans is a mycorrhiza-former with Larch. The chief interest of his paper lies however in his claim to having germinated the basidiospores of B. elegans. Melin (1922) had given a survey of previous unsuccessful attempts to germinate the basidiospores and he himself used Duggar's tissue culture method to isolate the fungus. The truth of Hammerlund's claim is somewhat doubtful, since his cultures were not completely sterile, nor was he able to obtain cultures from the germinated spores since these never developed further (cf.Melin 1924).

The purpose of the present investigation was to obtain more exact physiological data for B. elegans than were published by Melin, in the hope that these would lead to a clearer insight

into the relationship between the fungus and the host tree. It was also intended at the same time to determine how far B.elegans differs from other members of its genus in mycelial characters and physiological behaviour.

The work falls into two parts, first, an examination of the mycelium of B. elegans in pure culture; second an investigation of the relationship between pH value and growth on various media. This particular aspect of the growth relations of the fungus was chosen, partly because a knowledge of the reaction to pH value is essential before proceeding to other aspects of nutrition, and partly because the pH range is of interest in view of the acid soil conditions found in coniferous woods.

THE ISOLATION OF BOLETUS ELEGANS SCHUM.

Tissue culture is the only certain method for the isolation of fungi such as Boletus spp., the spores of which have rarely if ever been germinated.

Young sporophores, in which the annulus had not yet broken, were collected from a Larch plantation on October 11th, 1934. Each was cut off above the ground and placed in a sterilised specimen tube. The material was used on the day it was collected.

Mercuric chloride was employed as the sterilising agent, two concentrations being used:- 1% and 0.1%.

Each sporophore was placed in the sterilising agent for one minute, washed three times thoroughly in sterile distilled water and cut open with a sterile knife. A small segment was cut out of the pileus above the stipe and was transferred to a nutrient agar medium. This was made according to Melin's formula (1922):-

Glucose	20 gms
$MgSO_4 . 7H_2O$	0.1 gms
NH_4Cl	0.5 gms
KH_2PO_4	1.0 gms
Agar-agar	20.0 gms
Distilled water	1000 ccs

There were two series of isolations:-

Series A. Sterilised with 1% mercuric chloride
Series B. " " 0.1% " "

The inocula first began to develop 5 days after isolation.

It became apparent that sterilisation for 1 minute in 1% mercuric chloride was unnecessarily severe, since in Series A, 3 out of 6 inocula died, whereas in Series B all the inocula developed. Nevertheless there was no evidence that the previous treatment affected the growth of those inocula which did develop. There were cultures which grew vigorously in both series.

At first the cultures were subcultured alternately on glucose agar and beerwort agar, but later beerwort agar was used almost exclusively. In subculturing it was found necessary to transfer some of the old medium with the hyphae; a purely hyphal inoculum never developed. Melin (1925) found it was essential to

subculture at least once a month if vigorous growth in culture was to be maintained. The strains of Boletus elegans used in this investigation behaved similarly on the majority of media. As a result of subculturing once a month the vigour of growth of the stock cultures was maintained even after a period of two years.

PART I - MYCELIAL CHARACTERS.

Cultures have been examined at frequent intervals since the original isolation. Usually a thin slice of solid medium from a culture was first examined fresh and then fixed in 70% alcohol, stained in erythrosin (Gwynne-Vaughan & Barnes 1930) and mounted in glycerin-jelly. While this method was satisfactory for the routine examination of experimental cultures, it was found necessary to use cultures on gelatin slides for a more detailed examination of the hyphae. The following method was used:- (Sass 1929)

Agar-agar	0.75 gms
Gelatin	3.75 gms
Distilled water	250 ccs.

The sterile slides dipped in the above medium were moistened with a drop of nutrient solution and inoculated. After 4-6 days the cultures were examined fresh and later were fixed for 12 hours in:-

1% glacial acetic acid	40 ccs
Commercial formalin	10 ccs
95% Alcohol	50 ccs

The slides were washed and stored in 50% alcohol. The most satisfactory stain for the examination of hyphae was Delafield's haematoxylin. No cytological investigation was attempted.

Melin (1922) has described the mycelium of Boletus elegans growing in pure culture from Swedish material in the following terms:-

"Die Lufthyphen sind kräftig ausgebildet, anfangs weiss, spater mit rostgelben Flecken und schliesslich in alteren Kolonien ganz schmutzig rostgelb. Im Substrat bildet sich ein dunkelbrauner Farbstoff, der unter Umständen ziemlich weit von den Kolonie diffundiert.

"Die Fäden sind gleichförmig, ziemlich dünn, etwa 2-3,5μ dick, und gleichmässig septiert. Sie besitzen eine reichliche Versweigung, die Zweige gehen immer vom oberen Teil einer Zelle unmittelbar unter der Zellwand und unter eineim ziemlich konstanten Winkel (bis zu 45°) aus. Hyphenstränge enstehen nur in alten Kulturen. .....

.....

Schnallen kommen in den Kulturen nicht vor. Auch andere bei Pilzen vorkommende Fusionen treten sehr selten auf. Hauptsächlich sieht man bisweilen Zweigbrücken, so dass H-förmige Bildungen enstehen, aber nur ausnahmsweise Berührungsbrücken.

"Die älteren Lufthyphen sind dicht mit körnigen Ausscheidungen von sehr verschiedener Grösse belegt, so dass sie papillös erscheinen." (Melin 1922, p.173).

The English strain of B. elegans used in this investigation is similar in most respects to the Swedish strain described by Melin, the differences being largely those of degree. The English strain appears to be more vigorous in growth, thus whilst the diameters of the hyphae are 2μ - 5.5μ

in the English strain, they were only 2μ-3.5μ in the Swedish cultures. The chief points of interest are enumerated below.

Colour and General Appearance of Cultures

There appears to be very little difference in macroscopic appearance between the Swedish and English cultures. The rust yellow patches described by Melin are a feature of glucose media; on oat or beerwort agar, these are replaced by brown patches. It will be shown below that these dark coloured patches which eventually cover the whole colony are due to the presence of brown aerial hyphae. Under certain conditions which have not been determined cultures of B. elegans develop a bright yellow substance which diffuses into the surrounding medium. This yellow substance appears to be distinct from the brown substance which is mentioned by Melin and normally occurs in all cultures. When growth is vigorous drops of a brown oily fluid appear on the surface of the cultures.

The Branching of the Mycelium

There are two chief types of branching found in the mycelia of Boletus spp.

1. Simple branching, which Melin (see above) has described for B. elegans. Fig.1a.

2. "Paarige" branching, defined by Melin as follows:- "im oberen Teil der Zellen entspringen einander gegenüber zwei Zweige, wodurch ein armleutchterähnlicher Habitus zustande kommt." (Melin 1923, p.128).

While simple branching occurs in the mycelium of all

Boletus spp. so far investigated, 'paarige' branching is more limited in occurrence, and according to Melin simple branching is the only normal type in B. elegans. 'Paarige' branching occurs frequently in B. luteus and B. variegatus, and very infrequently in B. granulatus and B. badius.

In this investigation simple branching has been found to be most common in the mycelium of B. elegans, but 'paarige' branching occurs frequently in all cultures, on both the substrate and air hyphae, Figures 1b and 2. After two years' growth in culture there is no evidence that 'paarige' branching is becoming less frequent in occurrence or that it is confined only to the vigorous cultures as Melin found for M. radicis sylvestris and Boletus spp; although it certainly occurs less commonly in cultures showing poor growth.

The Occurrence of Clamp Connections

Melin (1923) classifies the species of Boletus cultured by himself into two classes:-

1. Species in which clamp connections occur more or less frequently, e.g. B. luteus and B. variegatus.
2. Species in which clamp connections are lacking or rarely observed, e.g. B. granulatus and B. badius.

B. elegans belongs to the second class in which clamp connections are rarely found; they were never observed by Melin and have only twice been observed in this investigation, Figures 3a, 3b.

Hammerlund (1923) reported the occurrence of clamp connections in a culture of basidiospores of B. elegans in formalin sterilised soil. No comparison with the cultures used by Melin and the writer is possible, however, since Hammerlund's cultures were probably not completely sterile and were derived from spores, and not from pieces of the sporophore. It is not improbable that the mycelium derived from germinating spores if it could be obtained in pure culture would differ in many respects from that obtained by tissue culture; but so far it has not been possible to compare them. However, it appears likely that, as Hammerlund thought, the occurrence of clamp connections is related to the vigour of growth of the cultures.

Hyphal Fusions

In contrast to the Swedish strain of B. elegans the English strain develops bridging hyphae frequently, especially among the hyphae on the surface of the substrate, Figures 4a, b and c. They are to be observed less frequently between the air hyphae.

Hyphal Strands

Strands 20 μ in diameter are commonly found in cultures on beerwort agar or oat agar after three weeks growth. Occasionally the English cultures have produced hyphal strands 45-46 μ thick which compare favourably with the strands figured

by Melin from cultures in symbiosis with larch seedlings; these were 40 µ in diameter. These differences serve to emphasise the greater vigour of the English strain.

Many of the strands examined consist of a central axis of wide hyphae containing glycogen and surrounded by a sheath of narrow hyphae, Figure 5. These strands show a similar type of organisation to that found in hyphal strands of other Hymenomycetes, e.g. Mushroom (Hein 1930), though the degree of differentiation is less, only two types of hyphal element being involved.

Brown Aerial Hyphae

Examination of the brown patches which appear after about 10 days' growth reveals the presence of numerous disconnected hyphal threads with yellow brown walls. These hyphae are mostly unbranched and always terminate abruptly with blunt end walls, forming relatively short threads. Occasionally hyphal bridges are observed between two such hyphae. The brown threads always occur on the outer surface of the mycelial mat; varying degrees in the intensity of the yellow brown colour are found, depending on the age of the culture, the older the culture, the darker the colour.

The brown hyphae appear to arise through a modification of the walls of the segments of normal hyaline air hyphae.

Occasionally it is possible to find a brown hypha continued at both ends into a normal hyaline hypha, Figure 7, but in most cases the unmodified portions of the hypha have shrivelled leaving the typical isolated brown fragments. The increasing depth of colour with age suggests the gradual deposition of the yellow brown substance in the walls.

Melin (1923) describes and figures hyphae in Mycelium radicis abietis which appear to be of a similar nature and are stated to be rich in glycogen. However, there appeared to be only traces of glycogen in the brown hyphae of B. elegans. Hyphae similar to those in B. elegans have been observed in large numbers in cultures of B. bovinus; it is probable that they are a general feature of Boletus spp. in culture.

Secretions

Apart from crystals of calcium oxalate which are formed on certain media, there are two kinds of secretion found regularly in cultures of B. elegans.

The first is characteristic of all Boletus spp. in culture. It consists of masses of granules covering certain of the aerial hyphae and hyphal strands, giving them the familiar papillated appearance, Figures 1 and 5. The granules are oily in nature; they form in greatest abundance on the vigorously growing cultures.

The second secretion consists of isolated brown crystalline masses which appear at the margins of cultures after three weeks' growth on glucose or beerwort medium. They occur in quantity in liquid beerwort media entangled in the hyphal mat. Their composition has not been determined.

The re-examination of the mycelium of B. elegans undertaken in this investigation has disclosed the existence of a greater similarity between the mycelia of this and other Boletus spp. than had been suggested by Melin. All the species of Boletus so far grown in culture show certain features in common, namely, 'paarige' branching, papillated hyphae, clamp connections and possibly 'brown' hyphae.

From Melin's work it seems that the species of Boletus can be classified into two groups on the extent to which their mycelia exhibit the above features. On the one hand are B. luteus and B. variegatus with frequent 'paarige' branching and numerous clamp connections; on the other hand are B. granulatus and B. badius with infrequent 'paarige' branching and few if any clamp connections. The Swedish strain of B. elegans would appear to belong to the latter group. It is possible that these two groups correspond to two fundamentally distinct growth types within the genus Boletus. It is more probable, however, that the type of mycelium obtained from any particular Boletus sp.

in pure culture is the result of the vigour of the particular culture, modifying to a great extent the effect of inherent characters. Thus the English strain of B. elegans is more vigorous in growth than the Swedish strain and in consequence shows features intermediate between the two groups, namely, the frequent occurrence of 'paarige' branching, combined with the almost complete absence of clamp connections.

Whether some species of Boletus are more difficult to grow in culture and will in consequence always exhibit the features of the second group rather than the first remains to be determined, but undoubtedly in B. elegans differences in mycelial branching and appearance are closely related to the vigour of growth in culture.

The mycelium of the strain of B. elegans used in this investigation does not appear to differ seriously in any respect from that isolated by Melin, nor is there any evidence that B. elegans exhibits features fundamentally different from the other Boletus spp. already known in pure culture.

All drawings were made with an Abbé camera lucida - magnification 460 diameters.

Fig.1. Strain 1½ yrs old. Air hyphae from 4 days old culture on glucose gelatin (a) simple branching
(b) 'paarige' "

(Drawing made from living material).

Fig.2. Strain 1 mth old. Part of air hyphae showing 'paarige' branching from 1 mth old culture on glucose agar.

Fig.3(a). Strain 1½ yrs old 'brown' hypha showing clamp connection from 16 days old culture on glucose agar.

(b). Strain 1¾ years old. Air hypha showing clamp connection from 2 mths old culture on beerwort agar.

Fig.4. Strain 1½ years old. Substrate hyphae showing hyphal fusions from a 6 days old culture on glucose gelatin.

Fig.5. Strain 1½ yrs old. Hyphal strands from a 2 mths old culture on beerwort agar.

Fig.6. Strain 1½ yrs old. Part of branched 'brown' hypha from 7 weeks old culture on oat agar.

Fig.7. Strain 1¼ yrs old. Immature 'brown' hypha from 2 mths old beerwort culture.

(a) Hyaline branch ~~belonging~~ beginning to change colour.

(b) Unchanged parts of original hyaline hypha.

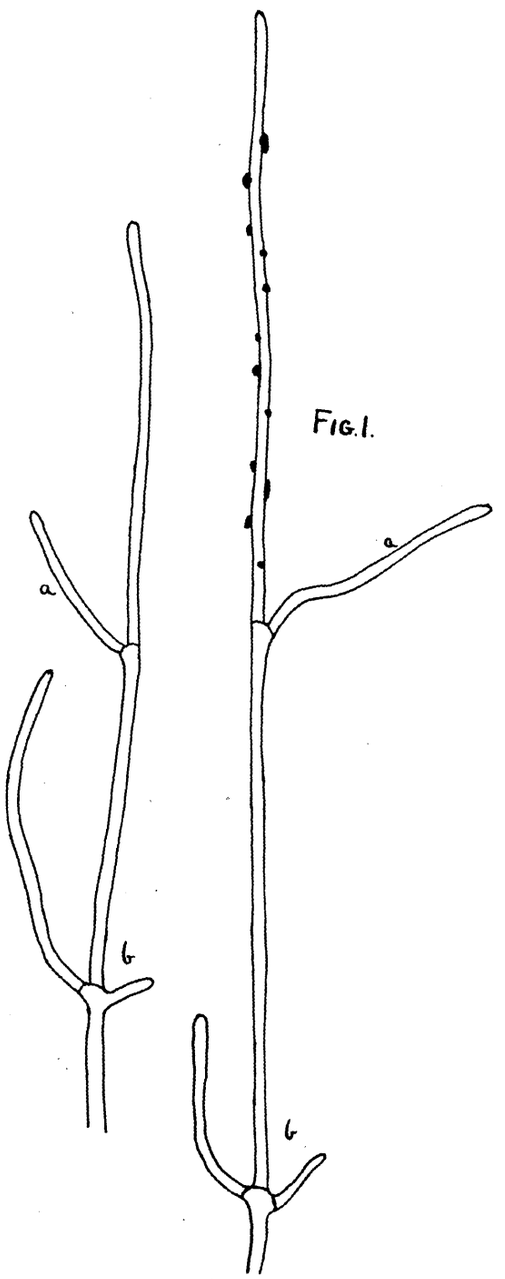
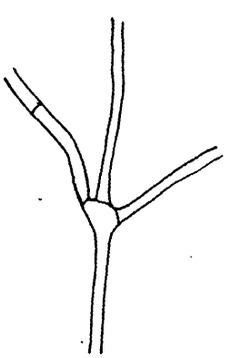


FIG. 1.

FIG. 2.



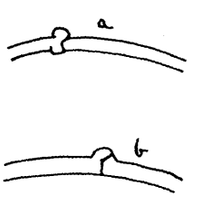


FIG. 3.

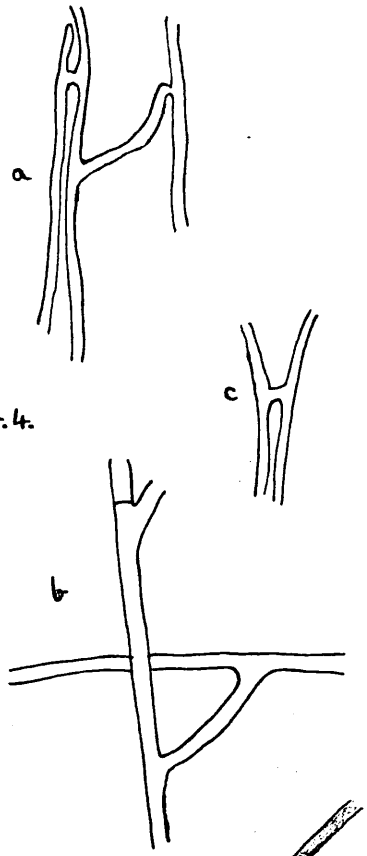


FIG. 4.

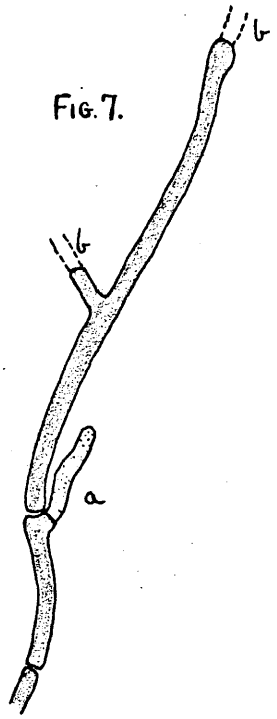


FIG. 7.

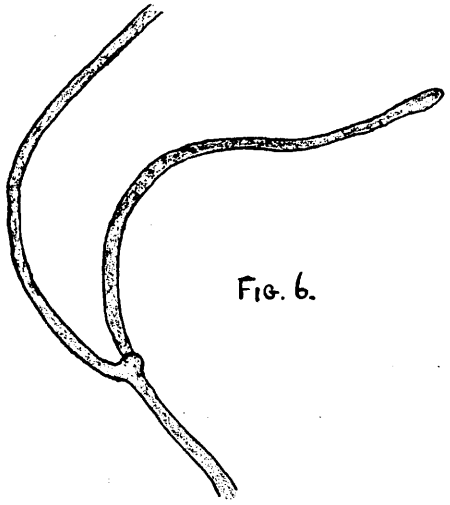


FIG. 6.

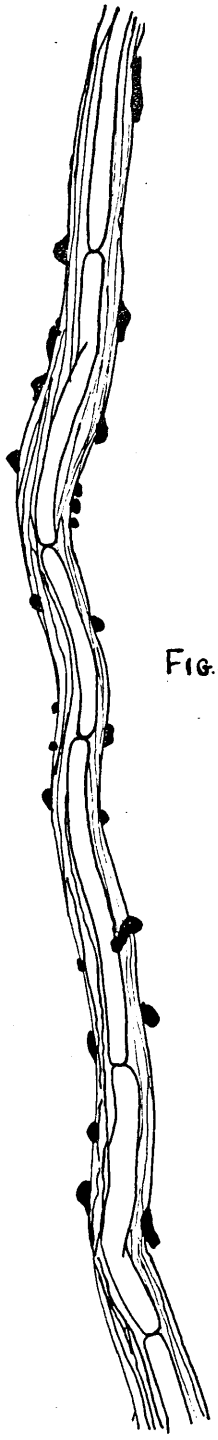


FIG. 5.

PART II. PHYSIOLOGICAL CHARACTERS

SECTION 1. EXPERIMENTAL PROCEDURE

(a) Inoculation of Cultures

It early became apparent that the initial condition of the inocula, that is to say the quantity of active hyphae contained in them, and the intensity of the staling products present in the substrate affected the rate of growth of the cultures and hence their final size. Thus two inocula, derived from a single culture and grown under the same conditions, at the end of three weeks differed in mean diameter by 1.3 cms. This difference is more than that often shown by cultures growing on two different media in one experiment; so that it was important to reduce the variations between inocula as much as possible. This was attempted in two ways.

First, wherever possible the average of 6 to 10 cultures, similar in origin, on one medium was compared with the average of the same number on another medium. In all experiments 10ccs of medium was used in petri dishes of standard size. Second, the differences between the initial condition of the inocula were reduced to a certain extent by using inocula of similar size. A punch was made for this purpose. The tip of a brass cork-borer 0.4 cms in diameter was soldered into a hole stamped out of the end of a metal scalpel. The cultures to be

used for inoculating experimental media were grown on various nutrient agars in petri-dishes. The cutter was sterilised with methylated spirit and discs of agar containing active growing hyphae were cut along the edge of the colonies. One such disc inoculum was placed with the hyphae downwards on the centre of each petri-dish. As far as possible each disc contained equivalent amounts of growing hyphae.

(b) The Measurement of Growth

Brown (1922,1923) has considered the relative merits of measuring the increase in diameter and the increase in dry weight when determining growth rate. He has pointed out that as a guide to the amount of mycelium present the method of linear measurement of necessity includes a large element of error. This arises from the varying types of growth obtained on different media; for example a 'fluffy' growth accompanied by much aerial mycelium, and a 'felted' growth with little aerial mycelium.

Despite this disadvantage, however, increase in diameter has been mainly used as the criterion of growth in these experiments for two main reasons. First, the colonies of B. elegans were fairly uniform in growth type in the experimental media studied, so that measurements of diameter did afford a reliable guide to the effect of a particular medium on the amount of mycelium formed; the density of the aerial mycelium was much

the same in all cases. Second, many difficulties were encountered when using liquid media. Melin (1925) mentioned that B. elegans is sensitive to aeration and hence does not grow well in liquid culture. Liquid media, if they are to be successful must be shallow, about 0.5 cms. in depth. Unless very large flasks are used the amount of medium is therefore small and the colony does not grow to any size. Since some agar had always to be carried over with the inoculum, staling products of the mother culture were of necessity transmitted to the liquid culture and would be present in significant concentration if the volume of liquid were small. These staling products diffused into the medium rapidly and occasionally prevented growth altogether. This condition was indicated in the medium by the presence of a distinct yellow colour derived from the inoculum. In one case an unbuffered nutrient solution of pH 6.8 became changed to pH 6.0 18 hours after the addition of an agar inoculum; no further change was observed. Moreover the presence of the agar introduced an error into the dry weights obtained. Often these were so small that it became necessary to weigh the mycelia from at least four cultures together and any error due to the differing amounts of agar could not be ignored. It was therefore felt that the increase in dry weight did not give as true a measure of the growth rate as the increase in diameter. The latter method was accordingly used in the majority of experiments.

The method of measuring increase in diameter in experiments Ia and Ib (Section 2) was not satisfactory. Growth of the cultures was often irregular, and circular colonies were often not obtained. Consequently the measurement of two diameters did not give a true estimation of the increase in size of the culture. Also, the growth was often so slow as to render it difficult to obtain an accurate determination by this method.

A new method was therefore devised. The image of the cultures, against a black background, was projected and drawn on paper by means of a specially modified epidiascope. This was adjusted to give an enlargement of the cultures to three times natural size. The areas of the resulting drawings were determined by means of a planimeter. The square roots of the values thus obtained when reduced three times were, for convenience, used as the diameters of the cultures; these values can be denoted by D'. If the actual diameters - D, of the cultures are required they may be calculated from the relation

$$D = .886D'$$

D' is the value used throughout the investigation - with the exception of Experiments Ia and Ib - and called the diameter.

(c) The Prevention of Infection

The experimental cultures were grown in small petri-dishes 5.5-6.0 cms in diameter, which were placed in envelopes

after inoculation and sealed. Each envelope had a circular window cut in the front and contained a sheet of black paper as a backing to the dish, so that the image of the culture was thrown up more clearly by the projector. The gum on the envelope was moistened with carbolic solution and the black paper slips were poisoned with 1% mercuric chloride in 95% alcohol.

(d) The Determination of pH Values

The pH values cited in this investigation were obtained colorimetrically. If 10 ccs or more of a medium were available a Lovibond comparator was used, if less than 10 ccs a B.D.H. capillator.

When the pH values at the margin of cultures on agar or gelatin media were required the following method was adopted. (Harley, [1934]). A ring of medium 1 - 2 mm in width was cut out round the edge of each colony and chopped finely with a sharp scalpel. The medium obtained was placed in a small glass container made by sealing one end of a glass tube 0.3 cms in diameter and 4-5 cms in length. A small cotton wool plug was fixed in the open mouth of the tube. A cork was fitted into one of the smaller rings of the cover of a water bath; into holes bored in this cork the tubes were fixed. They were heated over the water bath till the medium liquefied, when the pH values were obtained by the capillator method.

(e) Temperature

Unless otherwise stated the experimental cultures were kept in an incubator with the temperature controlled at 20° - 21°C.

SECTION 2. RELATION BETWEEN GROWTH AND THE VIGOUR OF THE MOTHER CULTURES.

Melin (1925) notes that the growth rate of B. elegans is affected by the age of the cultures when used for inoculation. This was confirmed in greater detail in Experiments Ia and Ib.

Experiment Ia. A glucose medium was used of the following composition:-

Glucose	1.8%
$MgSO_4 . 7H_2O$	0.01%
NH_4Cl	0.05%
KH_2PO_4	0.5%
Agar agar	2.0%

The medium was steamed for 20 mins on 3 successive days. Inoculation was carried out from 2 malt agar cultures by the disc method.

The petri-dishes were sealed on one side with adhesive tape. Two thin strips of paper were fixed, at right angles to one another, on the basal dish, the inoculum being immediately above their point of intersection. Two similar strips of paper were fixed on the upper dish vertically above the basal strips. Measurements were made by marking the limit of each culture on the upper strips of paper and thus two diameters at right angles were obtained. The temperature was not controlled.

The past history of the experimental cultures used in this experiment is shown below:-

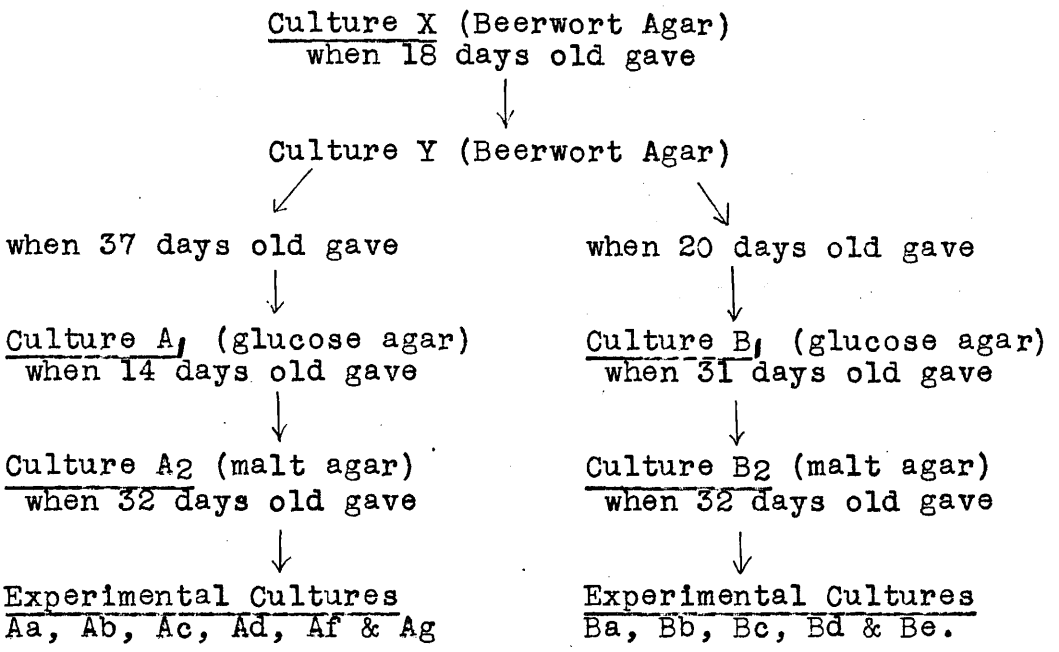


Figure 8 shows clearly that the cultures derived from culture A_2 were more vigorous than those derived from culture B_2. It is probable that the inocula~~tion~~ from culture A_1 used to start culture A_2 contained less staling products than the corresponding inoculum from culture B_1, used to inoculate culture B_2. This resulted in less vigorous growth in culture B_2 and hence the inocula derived from B_2 were less vigorous. That staling products present in the inoculum do diffuse into the medium and affect the rate of growth has been noted in Section 1.

FIG 8. AVERAGE DIAMETERS ON GLUCOSE MEDIUM. EXPERIMENT Ia.
TABLE I

– – – CULTURES Aa, Ab, Ac, Af, & Ag.

——— CULTURES Ba, Bb, Bc, Bd & Be.

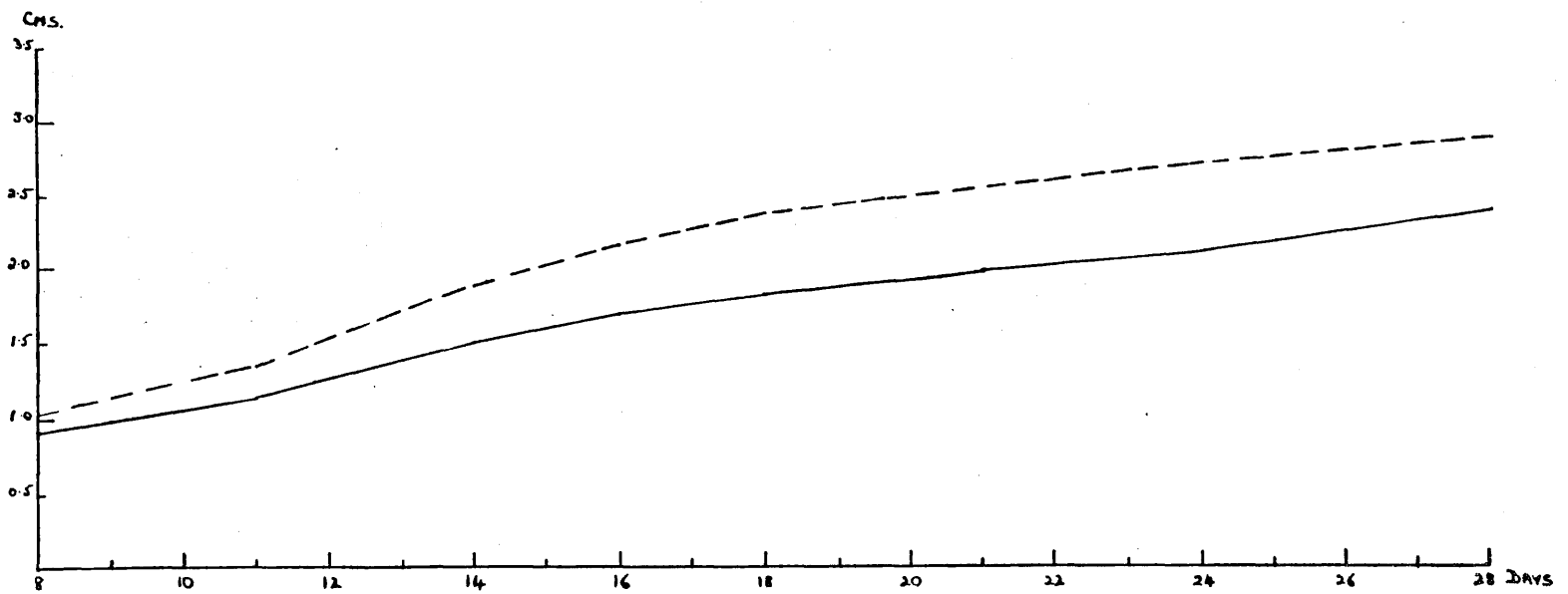


Table 1. Average Diameters of Cultures in Experiment 1a. Fig.8.

No. of days after inoculation	8	11	14	16	18	21	24	28
	cms	cms	cms	cms	cms	cms	cms	cms
Average diam. in cms of Aa, Ab, Ac, Af & Ag	1.03	1.34	1.87	2.15	2.37	2.55	2.72	2.89
Average diam. in cms of Ba, Bb, Bc, Bd & Be.	0.92	1.13	1.5	1.67	1.82	1.97	2.12	2.4

Experiment Ib. The method was similar to that used in the previous experiment, but 1.8% levulose replaced 1.8% glucose. Inoculation was again carried out from two malt agar cultures. The past history of the cultures was as follows:-

Culture X (Beerwort Agar) when 18 days old gave ↓

Culture Y (Beerwort Agar)

- when 20 days old gave ↓ Culture D1 (glucose Agar) when 31 days old gave ↓ Culture D2 (malt agar) when 40 days old gave ↓ Experimental Cultures Da, Db, Dc, Dd & Df
- Culture *C1 (Beerwort Agar) when 57 days old gave ↓ Culture C2 (malt agar) when 34 days old gave ↓ Experimental Cultures Ca, Cc, Cd & Ce

* Culture Y and Culture C1 were the same.

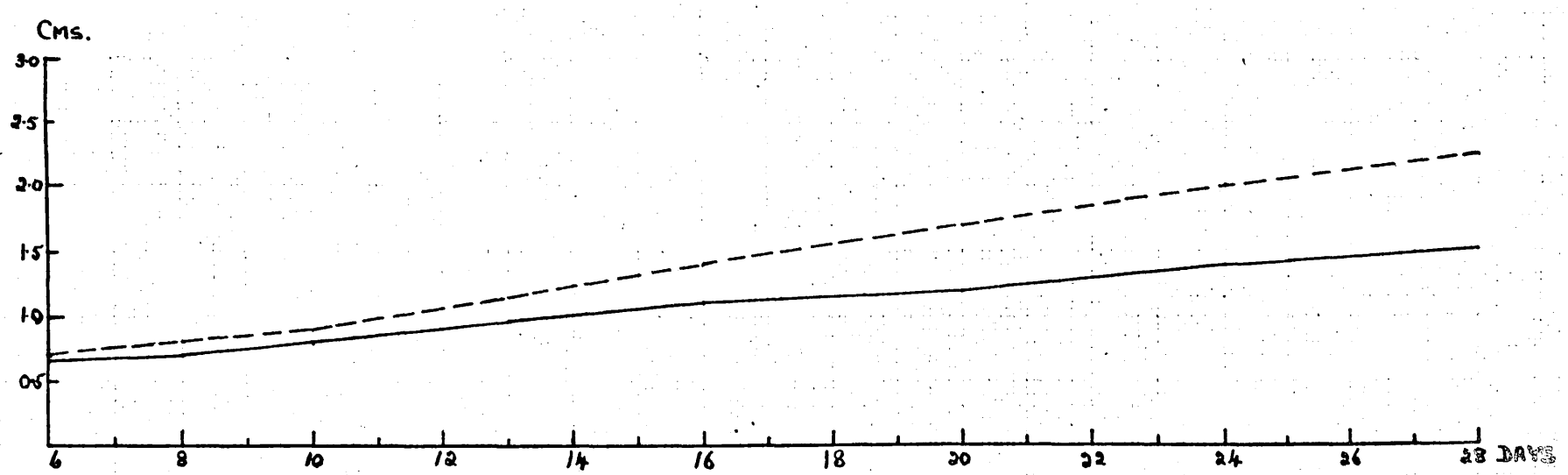
Figure 9 reveals the marked differences between the two sets of cultures. Cultures derived from culture C2 grew

FIG. 9. AVERAGE DIAMETERS ON LEVULOSE MEDIUM. EXPERIMENT I b.

TABLE 2.

___ CULTURES Ca, Cc, Cd, & Ce.

____ " Da, Db, Dc, Dd & Df.



more vigorously than those from culture D2, although culture C_1 was 57 days old when used and culture D_1 was only 31 days old. It appeared to be of more importance that culture C2 was only 34 days old when used while culture D2 was 40 days old. In this experiment the deciding factor appears to be the smaller quantity of staling products of the younger culture which masked the effects of its previous history. It should, however, be noted that culture D_1 was on glucose agar, while C_1 was on beerwort agar. Beerwort agar is generally a better medium for growth than glucose agar. It is, therefore, probable that the deleterious effect of the greater age of C_1 was partially overcome by the more vigorous growth possible on beerwort agar.

Table 2. Average Diameters of Cultures in Experiment Ib. Fig.9.

No. of days after inoculation	6	8	10	13	16	20	24	28
	cms	cms	cms	cms	cms	cms	cms	cms
Average diam. in cms of Ca, Cc, Cd & Ce	0.7	0.81	0.91	1.15	1.4	1.7	2.0	2.24
Average diam. in cms of Da, Db, Dc, Dd & Df.	0.65	0.71	0.8	0.97	1.11	1.2	1.4	1.5

Considering the experiments Ia and Ib together, it is evident that at least two generations prior to the experimental one, must be similar to ensure comparable results. This was

provided for in all later work, by inoculating experimental cultures from colonies which had been derived directly from stock culture.

It was found possible to obtain greater uniformity among cultures by using oat agar as the culture medium from which inocula for experimental purposes were taken. The formula of Lee Boner (1924) was used:-

Grind 60 gms Quaker Oats to a fine powder. Stir into 1000 ccs of cold water, add 10 gms agar-agar, heat till dissolved, pour and autoclave.

On oat agar growth was steady and uniform and continued vigorously for a much longer period than on any other medium; so that inoculation was possible when the cultures were 6-7 weeks old. Large petri-dishes were employed so that cultures were at their most vigorous stage when 4-5 cms in diameter. From a culture of this size as many as two dozen disc inocula could be cut and thus the number of different cultures involved in the inoculation of any one experiment was reduced, with a consequent reduction in the possible amount of variation shown by the experimental cultures.

Unfortunately, oat agar inocula are not suitable for liquid culture as they tend to fragment, giving a large number of small cultures in which varying amounts of absorbing surface influence the final result. In consequence, when liquid media were used the cultures were inoculated from beerwort agar.

SECTION 3. THE CHANGE IN pH VALUE DURING GROWTH.

Boletus elegans belongs to the class of organisms in which the direction of the change in pH value of the medium depends on the nutrient constituents, particularly the source of nitrogen (cf. Harley 1934). In this investigation two types of reaction have been observed to bring about a deflection in pH value; in one type, differential absorption of ions results either in increased acidity or in increased alkalinity, in the other type the diffusion of metabolic byproducts results in an increased alkalinity.

(a) Deflection in pH value due to differential absorption of ions.

(i) Towards greater acidity.

Experiment II. Source of nitrogen was NH_4NO_3

The medium contained the following constituents:-

Glucose	1.8%
$CaCl_2$	0.01%
$MgSO_4.7H_2O$	0.01%
NH_4NO_3	0.01%
KH_2PO_4	0.1%
Agar-agar	2%

Table 3a. Average Diameters of Cultures in Experiment II. Fig. 10.

No. of days after inoculation	2	4	7	9	13	16	21
Average Diam. in cms of 15 cultures	0.4	0.62	1.11	1.36	1.82	2.1	2.6

Table 3b. pH values at margins of Cultures in Experiment II. Fig.10.

No. of days	Diameter in Cms	pH Value	No. of days	Diameter in Cms	pH Value
2	0.38	5.6	9	1.34	4.4
2	0.39	5.2-5.4	13	1.78	4.0-4.2
4	0.69	4.6-4.8	14	1.95	3.8-4.0
4	0.7	4.6-4.8	16	2.12	3.6-3.8
6	1.0	4.4	17	2.19	3.6-3.8
7	1.09	4.2	21	2.68	3.6-3.8
8	1.18	4.4			
9	1.33	4.2			

Table 4. pH values of Liquid Media in Experiment III. Fig.11

No. of days after inoculation	0	7	9	13	21	29
pH values	4.8	4.8	4.8	4.8	5.0	5.8-6.0

The medium was autoclaved at 15 lbs pressure for 20 mins. The dishes were inoculated from two oat agar cultures 21 days old. At intervals two cultures were selected and the pH values at the margin of the cultures determined.

The relation between the diameters of the cultures and the pH values at their margins is shown in Figure 10, from which it appears that during growth the medium becomes increasingly acid.

(ii) Towards greater alkalinity

Experiment III Source of nitrogen KNO_3

A liquid medium in flasks was used of the following composition.

Glucose	1.8%
$MgSO_4.7H_2O$	0.01%
KH_2PO_4	0.1%
KNO_3	0.5%

The medium was autoclaved at 15 lbs pressure for 20 mins and inoculated from a beerwort culture.

The pH values of the medium in certain of the flasks were determined at intervals; the results are shown in Figure 11. Growth on a KNO_3 medium is very slow at first (see Section 6) hence the change in pH value is also slow; but throughout the experiment the tendency to increased alkalinity was apparent becoming very marked after 21 days.

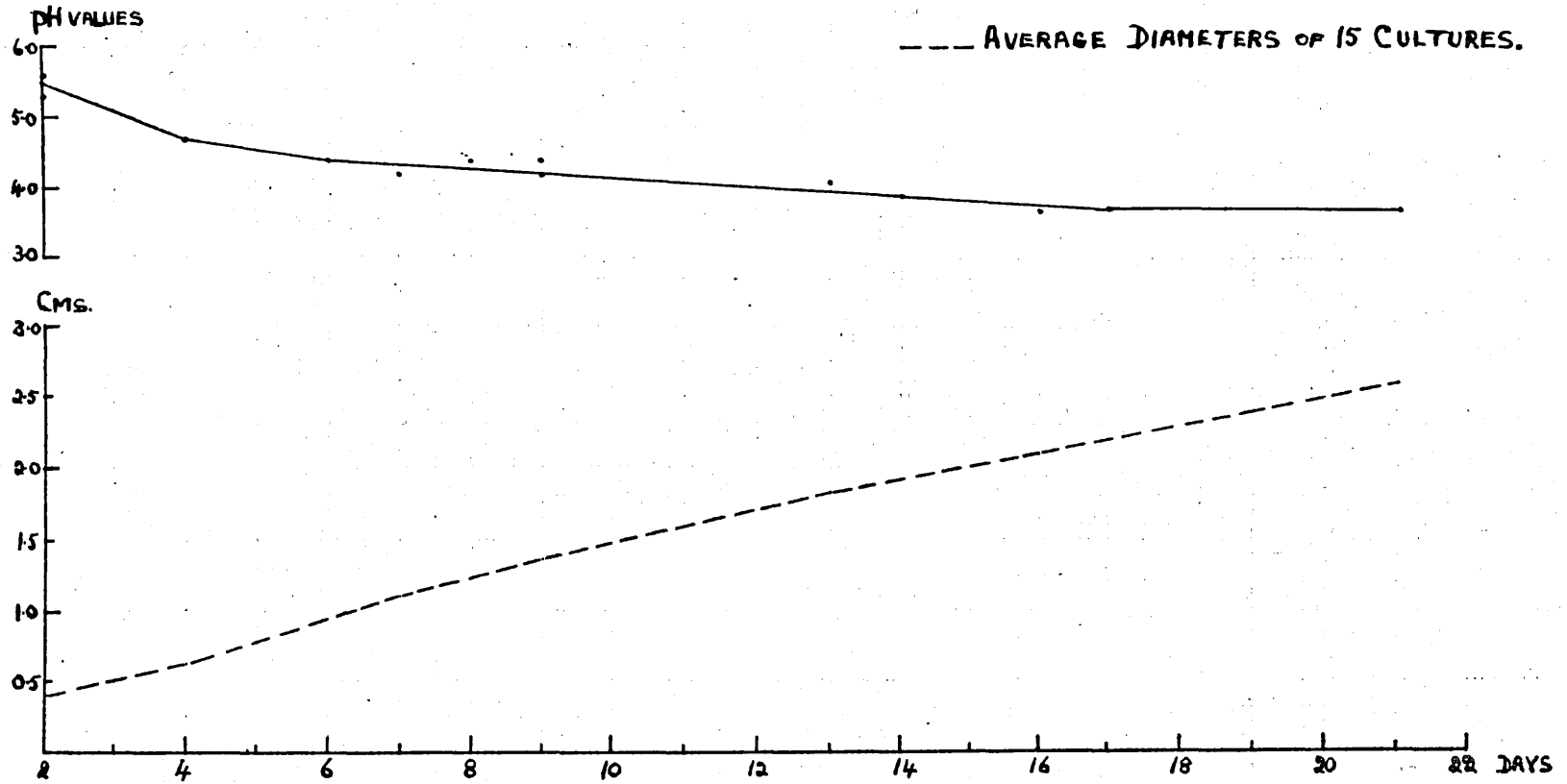
Since B. elegans is an ammonium organism the increase

FIG. 10. THE CHANGE IN pH VALUE ON AN AMMONIUM MEDIUM. EXPERIMENT II.

TABLES 30 & 36.

——— pH VALUES AT MARGINS OF CERTAIN CULTURES.

– – – AVERAGE DIAMETERS OF 15 CULTURES.



in acidity in Experiment II is to be expected, as the ammonium ions are absorbed leaving the nitrate ions in solution. In Experiment III where potassium nitrate was the only source of nitrogen the nitrate was absorbed leaving the potassium ions in solution. Thus the source of nitrogen determines the direction of the change in pH value of the medium for B. elegans.

(b) Deflection in pH Value due to Metabolic Byproducts

Experiment IV

The medium was as follows:-

Glucose	1.8%
$MgSO_4.7H_2O$	0.01%
NH_4Cl	0.05%
KH_2PO_4	0.1%
Gelatin	10%

The medium was sterilised by autoclaving for 20 mins at 15 lbs pressure. The dishes were inoculated from an oat agar culture 23 days old.

It will be seen from Figure 12 that during the first five days there was an increase in acidity, due no doubt to the absorption of ammonium ions leaving the chlorine ions in solution. After the fifth day the growth rate increased rapidly and simultaneously the pH value stopped decreasing and began slowly to increase.

It is suggested that these two changes occurred when the fungus began to liquefy the gelatin. During the process of

FIG. 11. THE CHANGE IN pH VALUE ON A NITRATE MEDIUM. EXPERIMENT III.

TABLE 4.

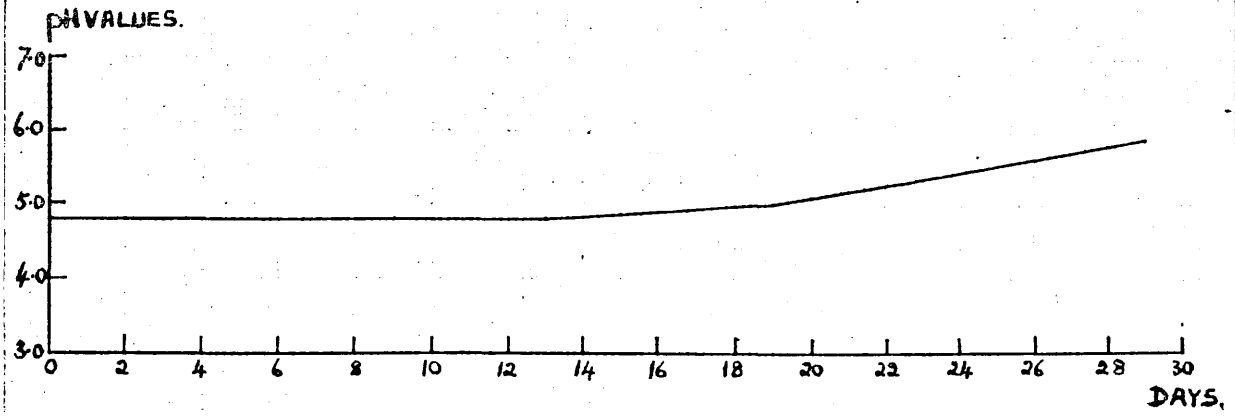
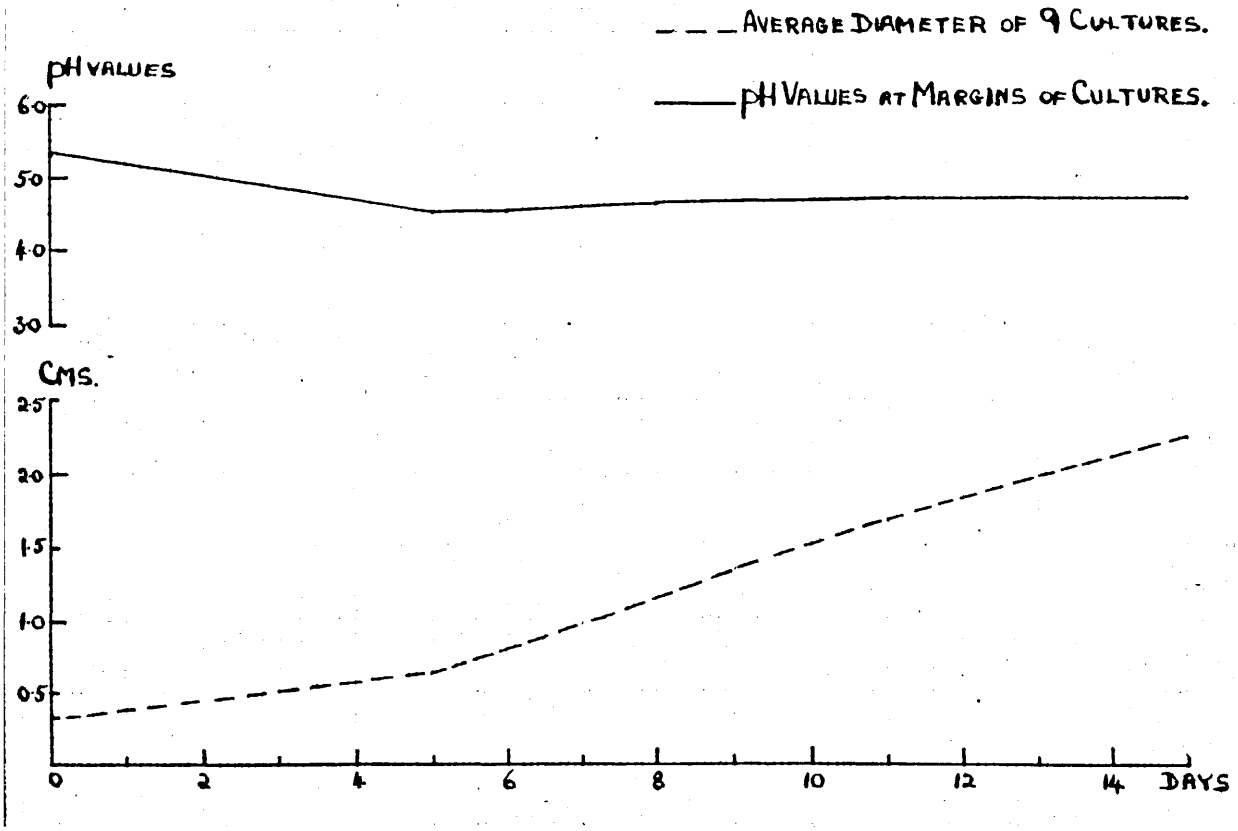


FIG. 12. THE CHANGE IN pH VALUE ON A GELATINE MEDIUM. EXPERIMENT IV.

TABLES 5a & 5b.



liquefaction the amount of available nitrogen increases with a resultant increase in growth rate, and the excess nitrogen diffuses into the substrate as ammonia - at first only sufficient to keep the pH value of the medium stable, but later in greater quantity leading to an increase in alkalinity.

This marked tendency shown by B. elegans to change the pH value of a medium during growth led to the conclusion that the pH range possible for growth could only be determined with certainty in strongly buffered media.

Table 5a. Average Diameters of Cultures in Experiment IV. Fig.12.

No. of days after inoculation	0	5	7	9	11	13	15
Average Diam. in cms of 8 cultures	0.35	0.63	0.97	1.34	1.67	1.98	2.25

Table 5b. pH Values at Margins of Cultures in Experiment IV. Fig.12.

No. of days after inoculation	0	5	6	8	11	15
Diam. in cms.....	0.35	0.64	0.75	1.19	1.68	2.26
pH Value	5.2-5.4	4.4-4.6	4.4-4.6	4.6	4.6-4.8	4.6-4.8

SECTION 4. THE INFLUENCE OF pH VALUE ON GROWTH IN GELATIN MEDIA.

All the experiments reported in this section were carried out with buffered media. A citric acid-phosphate buffer published by McIlvaine (1921) was chosen for this purpose, after investigating in two preliminary experiments whether

(a) citric acid can serve as a source of energy for B. elegans - Experiment V.

(b) citric acid exerts any influence on the growth rate of B. elegans in the presence of some other form of energy such as glucose - Experiment VI.

Experiment V.

The nutrient solution was as follows:-

0.1M citric acid	100 ccs
0.2M Na_2HPO_4	100 ccs
K_2SO_4	0.02 gms
NH_4Cl	0.2 gms

Half this solution was solidified with 2% agar, half was used in the liquid form. The pH value of both liquid and solid media was 4.8-5.0 after autoclaving for 20 mins at 15 lbs. pressure.

About 14 days after inoculation there were no signs of growth in the liquid medium. On the solid medium the inocula had sprouted, being clothed with a fringe of hyphae, but no growth on the medium could be detected. At the end of a month very slight growth had taken place in liquid media, but not on the solid media. While citric acid is not toxic to growth since the inocula sprouted, nevertheless it cannot serve as a source of energy for B. elegans.

Experiment VI

The following solutions were used:-

(i) Nutrient Solution.

Glucose	18 gms
K_2SO_4	0.1 gms
NH_4Cl	1.0 gms
Distilled water	250 ccs

(ii) Buffer Solution - pH 4.6

0.1 M citric acid	159.75 ccs
0.2 M Na_2HPO_4	140.25 ccs

The quantity of PO_4 radicle present in this solution was calculated to be 2.647 gms.

(iii) Phosphate Solution

$NaH_2PO_4 . 7H_2O$	6.408 gms
Distilled water	500 ccs

This solution contained an equivalent quantity of PO_4 radicle as solution 2, viz., 2.647 gms.

Two media were made up from these solutions.

Medium A. 100 ccs of solution (i) + 300 ccs of solution (iii)

" B. 100 ccs " " (i) + 300 ccs " " (ii)

Media A and B differed in respect of the concentration of Na^+ and OH^- ions and in the presence or absence of citric acid. The media were solidified with 10% gelatin, and sterilised by autoclaving for 15 mins at 15 lbs pressure. The pH value of both media after sterilisation was 4.8-5.0. Inoculation was carried out from malt agar cultures 21 days old. The temperature was not controlled during the experiment.

The pH values of the media at the margin of the cultures were determined at intervals throughout the experiment; there was found to be no change in pH value in either medium.

Table 6. The Average Diameters of Cultures in Experiment VI. Fig. 13.

No. of days after inoculation	6	9	12	15	18	21	25
Average Diam. in cms of 10 cultures on Medium A	0.38	0.55	0.76	1.02	1.3	1.53	1.91
Average Diam. in cms of 10 cultures on Medium B	0.38	0.53	0.72	1.02	1.37	1.58	2.0

The average growth rates of 10 cultures on each of the two media are compared in Table 6 and Figure 13. It is evident that citric acid in that concentration exerts no appreciable influence on the growth of B. elegans.

The range of pH values possible for the growth of B. elegans was determined in Experiments VIIa & VIIb.

Experiment VIIa

Three solutions were used.

1. 0.1M citric acid solution
2. 0.2M Na_2HPO_4 solution
3. Nutrient solution

Glucose	36 gms
K_2SO_4	0.2 gms
NH_4Cl	2.0 gms
Distilled water	500 ccs

In the culture media the buffer solution was diluted to three-quarters of the original concentration and the nutrient

FIG. 13. THE INFLUENCE OF CITRIC ACID ON GROWTH. EXPERIMENT VI

TABLE 6.

——— AVERAGE DIAMETERS ON MEDIUM A (WITHOUT CITRIC ACID).

– – – " " " " B (WITH CITRIC ACID).

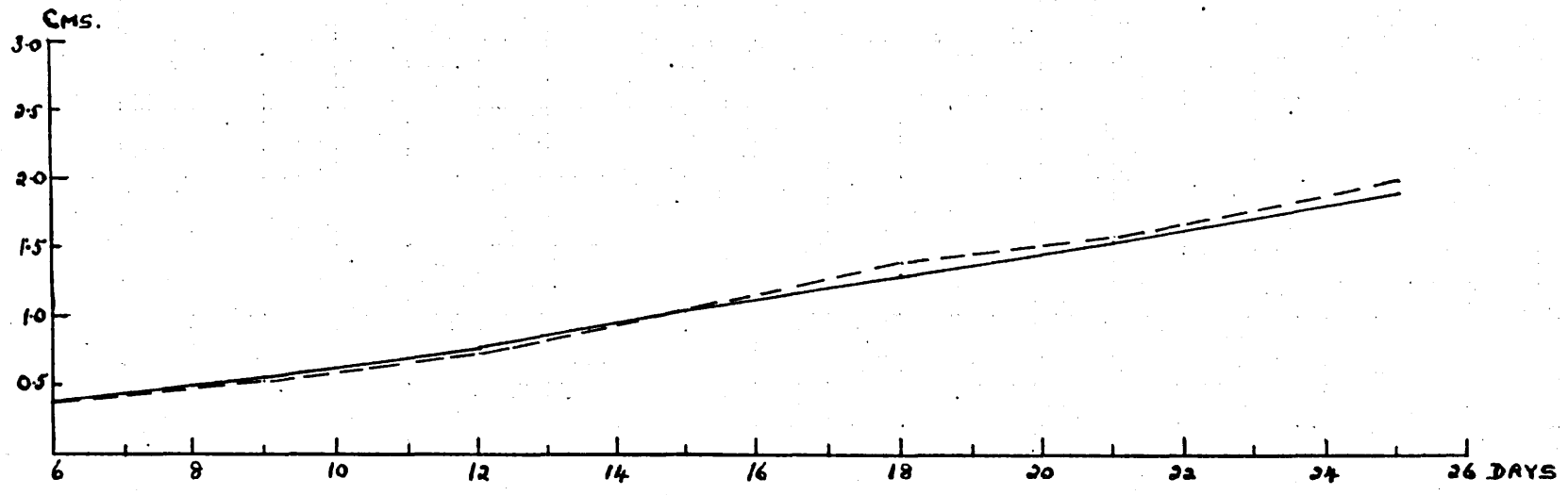


Table 7. The Composition of the Culture Media in Experiment VIIa.

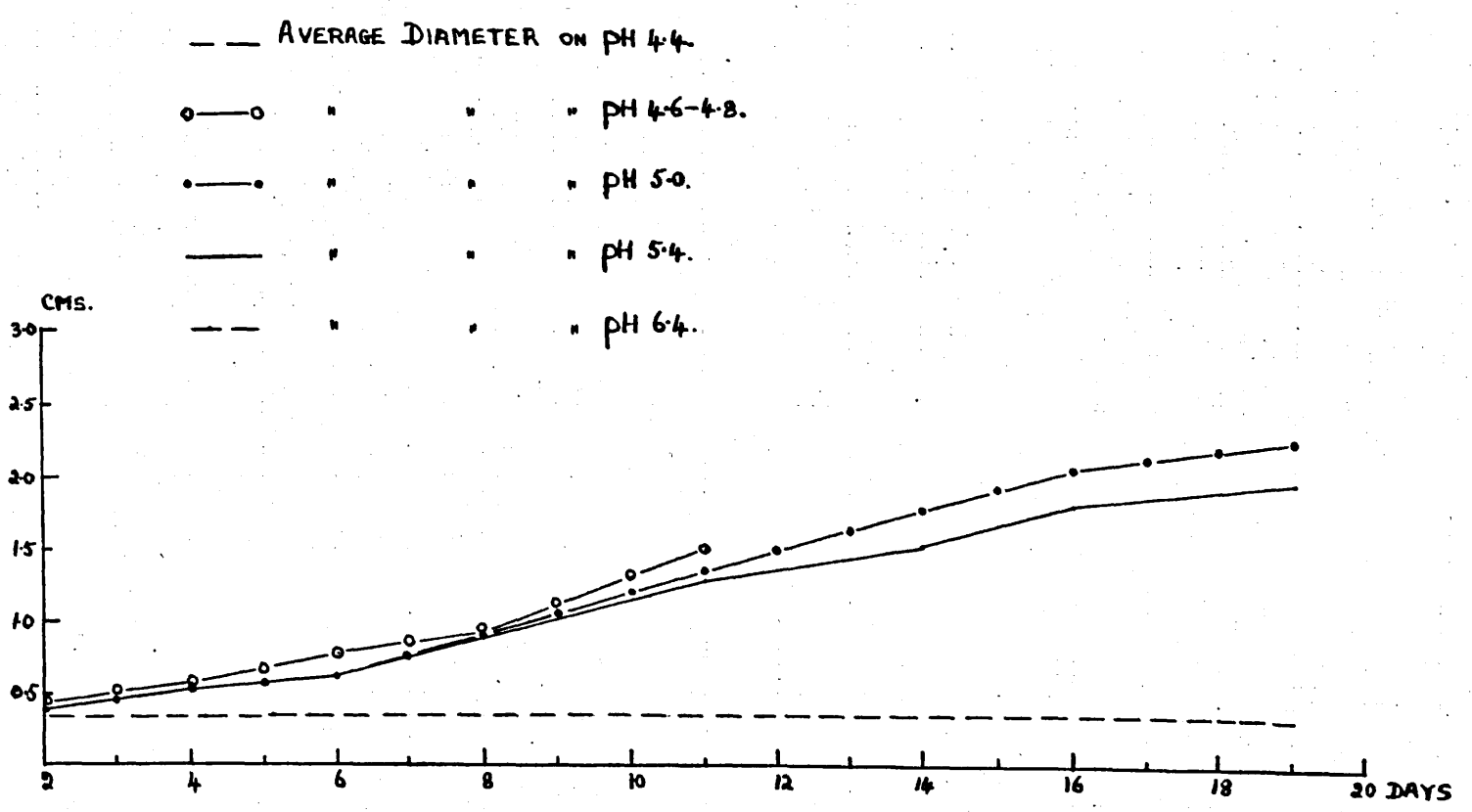
No.	pH Value before sterilisation	Quantity of 0.2M Na_2HPO_4	Quantity of 0.1M Citric acid	Quantity of Nutrient Solution	pH Value after sterilisation
		ccs	ccs	ccs	
1	3.6	36.99	143.01	60	4.4
2	4.0	69.39	110.61	60	4.6-4.8
3	4.8	88.74	91.26	60	5.4
4	5.6-5.8	104.4	75.6	60	5.4
5	6.8	130.95	49.05	60	6.4

Table 8. The Average Diameters of Cultures in Experiment VIIa. Fig 14.

pH values	pH 4.4	pH 4.6-4.8	pH 5.0	pH 5.4	pH 6.4
No. of cultures averaged		4	5	8	
No. of days after inoculation					
	cms	cms	cms	cms	cms
2	0.35	0.42	0.39	0.39	0.35
4	"	0.57	0.52	0.53	"
6	"	0.78	0.61	0.62	"
8	"	0.92	0.9	0.88	"
11	"	1.51	1.34	1.29	"
14	"	-	1.77	1.52	"
16	"	-	2.07	1.83	"
19	"	-	2.29	2.0	"

FIG. 14. THE RELATION BETWEEN GROWTH AND pH VALUE ON GELATINE MEDIA. EXPERIMENT VIIa

TABLE 8.



solution to one quarter. 10% gelatin was added to each of the culture media, which were steamed for 30 minutes on two successive days and 20 minutes on the third day. The pH values were determined after sterilisation - cf. Table 7. The culture media were inoculated from 3 oat agar cultures 43 days old.

The average diameters of the experimental cultures are given in Table 8. Tests of the media at the end of the experiment indicated that no change in pH value occurred during growth. The values in Table 8 are set out in Figure 14, where it appears that the range of pH value possible for growth lies between 4.4-4.8 and 5.4-6.4.

Experiment VIIb

The procedure was as far as possible similar to that in Experiment VIIa. Media of slightly different pH value (cf.Table 9) were employed and were steamed for 20 mins on two successive days and for 20-30 mins on the third day. 4 oat agar cultures 25 days old were used for inoculation.

The growth rates obtained are tabulated in Table 10 and shown graphically in Figure 15. From this experiment it appears that the range of pH value possible for growth lies between 5.0-5.2 and 6.0+.

The results of Experiments VIIa & VIIb have been compared in Figure 16, which is based on the average diameters which would have been obtained had all the inocula been 0.39 cms in diameter 3 days after inoculation - see Table 11.

Table 9. The Composition of the Culture Media in Experiment VIIb.

No.	pH Value before sterilisation	Quantity of 0.2M Na_2HPO_4	Quantity of 0.1M Citric acid	Quantity of Nutrient Solution	pH Value after sterilisation
		ccs	ccs	ccs	
1	4.0	69.39	110.61	60	4.6-4.8
2	4.4-4.6	79.38	100.62	60	4.8-5.0
3	5.0	92.7	87.3	60	5.2
4	5.6-5.8	104.4	75.6	60	5.6
5	6.2	113.67	66.33	60	5.8-6.0

Table 10. The Average Diameters of Cultures in Experiment VIIb. Fig.15.

pH Value	pH 4.6-4.8	pH 4.8-5.0	pH 5.2	pH 5.6	pH 5.8-6.0
No. of cultures averaged			10	10	8
No. of days after inoculation					
	cms	cms	cms	cms	cms
3	0.35	0.35	0.35	0.43	0.37
5	"	"	0.42	0.54	0.47
7	"	"	0.67	0.82	0.5
10	"	"	1.12	1.28	0.86
12	"	"	1.48	1.61	1.07
14	"	"	1.91	1.85	1.28
17	"	"	2.36	2.36	1.44
19	"	"	2.69	2.59	1.68
21	"	"	3.01	2.88	1.81

FIG. 15. THE RELATION BETWEEN GROWTH AND pH VALUE ON GELATINE MEDIA. EXPERIMENT VII b

TABLE 10.

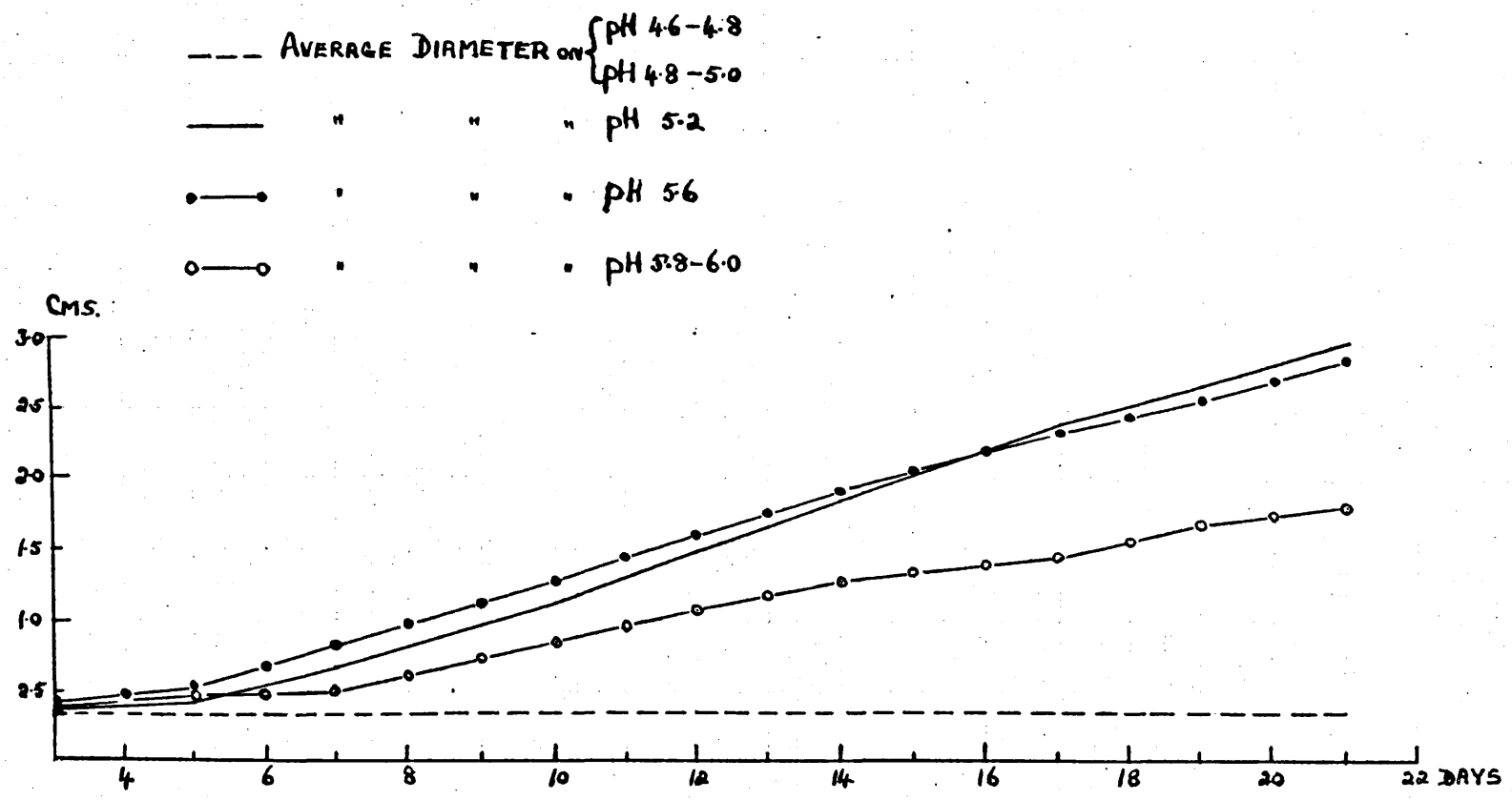


Table 11. Average Diameters of Cultures 19 days after Inoculation in Experiments VIIa & b. Fig. 16.

(Calculated on the basis that all cultures were 0.39 cms in diameter 3 days after inoculation.)

Experiment VIIa		Experiment VIIb	
pH Value	Av. Diam. in Cms	pH Value	Av. Diam. in Cms.
4.4	0	4.8-5.0	0
4.6-4.8	2.39	5.2	2.4
5.0	2.29	5.6	2.35
5.4	2.0	5.8-6.0	1.78
6.0	0		

It is apparent that the growth reaction of B. elegans to the pH value of the medium was different in these two experiments, in Experiment VIIa growth was possible on pH 4.6-4.8, in Experiment VIIb growth was not even possible on pH 4.8-5.0.

A possible explanation of the discrepancy was sought in the molecular concentration of the solutions - since this was known to exert an influence in certain cases (cf Section 7). Examination of Table 12 however does not support this view. The molecular concentrations decreased with increasing acidity, hence the most acid media were the least toxic. Also, the molecular concentration is the same in both the media of pH 4.6-4.8, in Experiments VIIa & VIIb, yet no growth occurred in media of that

FIG. 16. THE RELATION BETWEEN GROWTH AND pH VALUE IN GELATINE MEDIA. EXPERIMENT VIIa & b. TABLE II.

____ CULTURES IN EXPERIMENT VIIa.

_ _ _ _ " " " VIIb.

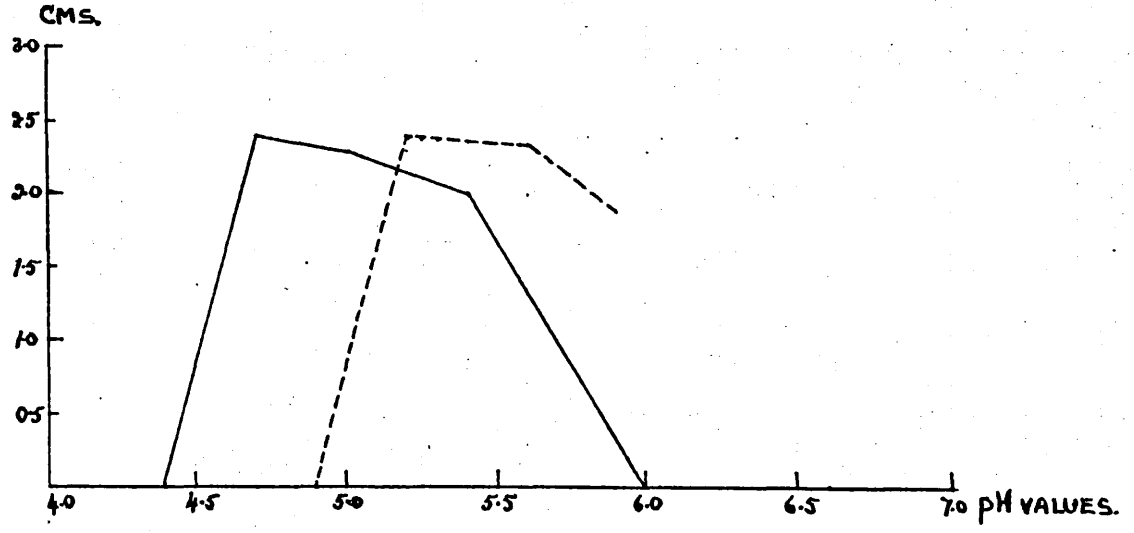


Table 12. The Molecular Concentrations of the Media used in Experiments VIIa & VIIb.

Experiment	pH Value after sterilisation	Mol. Conc. of Nutrient	Mol. Conc. of Na_2HPO_4	Mol. Conc. of Citric Acid	Total Mol. Concentration	Reaction of *B. elegans*
VIIa	4.4	0.12 M	0.031 M	0.06 M	0.211 M	No growth
	4.6-4.8	0.12 M	0.058 M	0.046 M	0.224 M	growth
	5.0	0.12 M	0.076 M	0.038 M	0.234 M	"
	5.4	0.12 M	0.087 M	0.031 M	0.238 M	"
	6.4	0.12 M	0.12 M	0.02 M	0.26 M	No growth
VIIb	4.6-4.8	0.12 M	0.058 M	0.046 M	0.224 M	No growth
	4.8-5.0	0.12 M	0.066 M	0.042 M	0.228 M	"
	5.2	0.12 M	0.077 M	0.036 M	0.233 M	growth
	5.6	0.12 M	0.087 M	0.031 M	0.238 M	"
	5.8-6.0	0.12 M	0.095 M	0.028 M	0.243 M	"

composition in Experiment VIIb, although growth was vigorous in VIIa. Evidently differences in the molecular concentrations of the solutions played no part in determining these results.

During sterilisation of acid gelatin and agar solutions there is a deflection of the pH value in the direction of greater alkalinity. It is possible that the changes in the media brought about by the hydrolysis of the gelatin affect the position of the limiting pH values for the growth of B. elegans. This was investigated in Experiment VIII.

Experiment VIII

Solutions similar to Nos. 2 & 3 in Expt. VIIa and Nos. 2 & 3 in Experiment VIIb (see Tables 7 & 9) were used. 10% gelatin was added to each medium; it was dissolved by gentle warming so that as little change as possible occurred in the media. When liquefied the media were tubed and steamed for exactly half an hour and left to cool, 3 tubes of each medium being sloped. This process was repeated for two further half hours and a final one hour. The tubes were inoculated from a 23 day old oat agar culture and the colonies were measured 13 days after inoculation.

A second part of the experiment was carried out at a later date. Portions of the stock culture media IIIA and IIID (see Table 13) after being reliquefied to give IIIAx and IIIDx,

were mixed in equal proportions to give (A + D). IIIAx, IIIDx and (A + D) were steamed for half an hour and inoculated from the original oat agar culture which was then 6 weeks old. *

The results are shown in Table 13 and may be accounted for in the following manner.

It appears that in gelatin media of this composition B. elegans is very sensitive to the pH value of the medium. The slight decrease in acidity resulting from steaming for 1 hour compared with steaming for half an hour in series A enables growth to occur in 2 out of 3 cultures. In series B the first two media were obviously near the limiting acidity since all the inocula did not grow; but after steaming for 1½ hours this effect disappeared and all the inocula germinated. This condition in which only some of the inocula in a medium germinate had appeared also in Experiment VIIa, where on pH 4.6-4.8 only 4 out of 12 inocula grew. There is a definite indication that an increased growth rate in series A results from decreased acidity. In series B no such alteration in growth rate is perceptible. In series C, however, steaming for 1 hour gives a better medium for growth than ½ hour or (1½-2½) hours; thus there appears to be an optimum pH value between pH 5.0 and pH 5.2. On medium D,

* There is no evidence that these later inocula were less vigorous.

Table 13. Area in Sq. Cms of Cultures 13 Days after Inoculation in Experiment VIII.

	Time of Sterilisation	I ½ hr	II (½+½) hrs	III (½+½+½) hrs	IIIx (½+½+½+½) hrs	IV (½+½+½+1) hrs
A	pH after sterilisation	pH 4.6	pH 4.6	pH 4.6-4.8	pH 4.8	pH 4.8
	Area of individual cultures	0, 0, 0	0, 1.3,1.3	1.5,1.3,1.1	2.1,1.6 -	xx
	Average area	0	1.3	1.3	1.85	
B	pH after sterilisation	pH 4.6-4.8	pH 4.6-4.8	pH 4.8		pH 4.8-5.0
	Area of individual cultures	0, 2.2,2.0	0, 0, 2.1	1.8,1.7,2.9		xx
	Average area	2.1	2.1	2.1		
ø (A+D)	pH after sterilisation				pH 5.0	
	Area of individual cultures				2.2,1.9,2.8	
	Average area				2.3	
C	pH after sterilisation	pH 5.0-5.2	pH 5.0-5.2	pH 5.2		pH 5.2
	Area of individual culture	1.9,1.9,2.2	2.9,2.7,2.7	1.6 - -		1.6,1.6 -
	Average area	2.0	2.8	1.6		1.6
D	pH after sterilisation	pH 5.0-5.2	pH 5.0-5.2	pH 5.2	pH 5.2	pH 5.2-5.4
	Area of individual culture	3.0,3.2,3.1	2.7,2.5, -	1.7,2.3,1.8	2.1,1.9 -	1.2,1.4, -
	Average area	3.1	2.6	1.9	2.0	1.3

ø inoculated 3 weeks after the other media.

xx media did not gel; inocula grew below the surface.

with increased steaming decreased growth results, again the optimum occurs between pH 5.0 and pH 5.2. Throughout this experiment the changes in pH value were so slight after each steaming that it was difficult to register them using a colorimetric method.

While the delicate adjustment between pH value and growth furnished a sufficient explanation of these results the possibility that toxic substances formed during hydrolysis also played a part in determining the growth reaction could not be excluded. If such toxic substances were formed, the mixing of Media IIIA and IIID should result in growth as poor as that obtained from either of them alone. Actually growth was better on (A+D) than on either IIIAx or IIIDx; this is in accord with the better pH value of (A+D) namely 5.0. Hence, if any toxic substances were present their effect was masked by the more beneficial pH value of (A+D), (cf. Experiment X, where there is evidence for the existence of toxic products).

In Table 14 the results from Experiments VIIa and VIIb are compared with those from Experiment VIII. With the one exception of medium B (Experiment VIIb) the growth reaction corresponds with the amount of sterilisation. Since, however, medium B in Experiment VIIb and medium B in Experiment VIII were made up on different occasions, they were not exactly similar and this would account for the discrepancy (see Experiment X). Also it is probable that a longer steaming of

medium in Experiment VIIb would have rendered it less acid and more capable of supporting the growth of B. elegans.

It can therefore be concluded that B. elegans is highly sensitive to pH value in gelatin media of this composition. The minimum value for growth lies just below pH 4.8, the optimum value about pH 5.0.

Table 14. Comparison of Growth Reaction in Gelatin Media of pH 4.6-5.0. Expts. VIIa & b, VIII.

Medium	Expt.	No. of hrs Steaming	pH Value	Growth Reaction
A	VIII	$\frac{1}{2}$ hr	4.6	No growth
	VIIb	$(\frac{1}{2}+\frac{1}{3})$hr	4.6-4.8	" "
	VIIa	$(\frac{1}{2}+\frac{1}{2})$hr	4.6-4.8	$\frac{11}{12}$ grew
	VIII	$(\frac{1}{2}+\frac{1}{2})$hr	4.6	$\frac{2}{3}$ grew
	VIII	$(\frac{1}{2}+\frac{1}{2}+\frac{1}{2})$hr	4.6-4.8	$\frac{3}{3}$ grew
B	VIII	$\frac{1}{2}$ hr	4.6-4.8	$\frac{2}{3}$ grew
	VIIb	$(\frac{1}{2}+\frac{1}{3})$hr	4.8	No growth
	VIII	$(\frac{1}{2}+\frac{1}{2})$hr	4.6-4.8	$\frac{1}{3}$ grew
	VIII	$(\frac{1}{2}+\frac{1}{2}+\frac{1}{2})$hr	4.8	$\frac{1}{3}$ grew

A comparison with the results obtained above from buffered liquid media of similar composition suggests that the position of the minimum pH value is affected by the presence of gelatin.

Experiment IX

The composition of the media is shown in Table 15.

Table 15. The Composition of the Culture Media in Experiment IX.

pH value before sterilisation	Quantity of 0.2M Na_2HPO_4	Quantity of 0.1M citric acid	Quantity of Nutrient Solution	pH value after sterilisation
	ccs	ccs	ccs	
4.6	84.15	95.85	60	4.6
5.0	92.7	87.3	60	5.0
5.4-5.6	104.4	75.6	60	5.6

30 flasks (100 ccs capacity) each containing 10 ccs of medium were used. After autoclaving for 20 minutes the media were inoculated from 3 beerwort agar cultures 29 days old. After 21 days the dry weights were determined. Since the weight of a single culture was known to be very small, and the original agar discs were of unknown weight, the weight of 10 cultures taken together was determined. The contents of 10 flasks in each set were filtered through a weighed filter paper, the residues washed six times with distilled water and dried at 80° C until constant in weight.

The results obtained are recorded in Table 16. There was no growth of any of the inocula in the medium pH 5.6, so that the weight obtained was that of the 10 agar discs.

Table 16. Dry Weights of Cultures in Experiment IX.

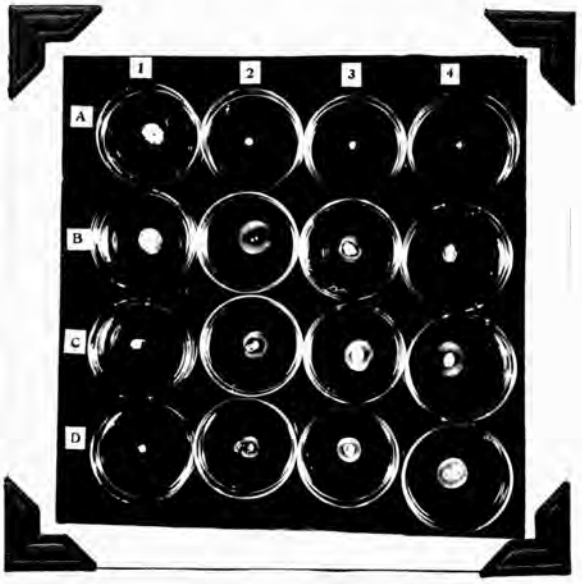
pH value	Weight of 10 cultures plus weight of agar	Weight of 10 cultures less weight of agar	Average weight of 1 culture
4.6	0.0558 gms	0.0458 gms	4.58 mgms
5.0	0.0214 gms	0.0114 gms	1.14 mgms
5.6	0.01 gms	0	0 mgms

It is evident that growth is better at pH 4.6 than at pH 5.0. Therefore the minimum pH value is below pH 4.6. The addition of 10% gelatin results in the deflection of this minimum pH value to pH 4.8. This relation between the presence of gelatin and the minimum pH value was investigated further by determining the effect of the concentration of gelatin on the pH range in buffered solutions.

Experiment X .

The solutions used were similar to Nos. 2 & 3 in Experiment VIIa and Nos. 2 and 4 in Experiment VIIb, (see Tables 7 & 9). Each solution was used in liquid form, and also with the addition of 3%, 6% and 10% gelatin. After steaming for 40 mins the pH values were determined in samples from all the media. The cultures were grown in petri-dishes inoculated from 2 oat agar cultures 25 days old. The inocula were supported on watch glasses in the liquid media, so that growth was on the surface of the substrate in all cultures. A photograph of the cultures 13 days after inoculation is shown in Figure 17.

Fig. 17. Photograph of Cultures in Experiment X. Table 17.



The areas of the cultures at that time are shown in Table 17.

Table 17. Increase in Area in Sq cms of Cultures in Experiment X. Fig.17.

(13 days after inoculation)

		1	2	3	4
		0 gelatin	3% gelatin	6% gelatin	10% gelatin
A	No. 2 in Experiment VIIa	pH 3.8-4.0 * 1.11	pH 3.8-4.0 * 0	pH 4.2-4.4 0	pH 4.4 0
B	No. 2 in Experiment VIIb	pH 4.4 * 1.33	pH 4.4 2.44	pH 4.6 1.31	pH 4.8 0.3
C	No. 3 in Experiment VIIa	pH 4.8 * 0.18	pH 4.8 2.39	pH 5.0 2.42	pH 5.0 2.5
D	No. 4 in Experiment VIIb	pH 5.2-5.4 * 0.1	pH 5.4 1.46	pH 5.4 1.52	pH 5.4-5.6 2.0

* Media in liquid state.

This experiment indicates that both the position of the pH range and the pH value in which greatest growth occurs are deflected towards less acid values in the presence of gelatin, the extent of the deflection depending on the percentage of gelatin.

The addition of gelatin to a nutritive acid medium must introduce many new factors, some of which may affect growth. Among these factors three should be noted in particular, the alteration of the physical state, the increase in nutritional value, and the addition of the products of gelatin hydrolysis. Firstly, the change in physical state from liquid to solid does not appear to have been of any importance in this experiment; for solutions A_1 and A_2 were both liquid, with the same pH value, but no growth occurred in A_2, while growth was vigorous in A_1. Secondly, the increase in available nitrogen could have produced the results in Series C and D but not in Series A or B; it is therefore ruled out as the sole determining factor, though undoubtedly it must affect the growth in some measure.

It is difficult to assess accurately the effect of the hydrolysis of the gelatin, since nothing appears to be known of the rate and extent of the process in solutions of the acidity used in these experiments, viz. pH 4.0-5.6. Presumably, however, the greater the acidity the greater is the extent of the hydrolysis; and with a given acidity, the higher the concentration of the gelatin used the greater is the quantity of hydrolysis products formed. It follows in Experiment X that the

concentration of hydrolysed gelatin decreases from Series A to Series D, but within each series the concentration increases from 1-4. This fact taken in conjunction with the effect of pH value and the varying nutritional value of the media appears to furnish the clue to these results.

In buffered media of this particular molecular concentration (see Table 12) the pH range for B. elegans lies between 3.8-4.0 and 5.6, with the optimum value in the region of 4.6 (see also Experiment IX). The effect of any toxic substance would be to shorten this range, while favourable factors would lengthen it. The growth reaction of B. elegans in Experiment X indicates that during the hydrolysis of gelatin some toxic product is formed. In Series A the toxic product is sufficiently concentrated to inhibit growth at pH 4.4 . In Series B it is not so concentrated and the pH value is nearer the optimum, hence growth is possible; growth is better in B_1 than in B_2 owing to the increased nitrogen content of the former, but B_3 and B_4 contain greater quantities of hydrolysed gelatin than B_2 and growth is thereby retarded. In Series C and D the toxic substance is still less concentrated and ceases to affect growth. Series C has a more favourable pH value than Series D and hence growth is better in the C cultures than in the corresponding D cultures. Within each of Series C and D the increasing nutritional value of the media with increasing

concentrations of gelatin would account for the difference in size of the cultures.

It has not been possible to determine how far the above analysis is correct. When gelatin which had been completely hydrolysed in strong acid was added to solution A in varying amounts it was found that increasing quantities of hydrolysed gelatin retarded growth up to 3% approximately, above this, growth was completely inhibited. Since the products present in a sample of gelatin completely hydrolysed in strong acid are probably very different from those present after steaming with very weak acid, this experiment affords no proof of the presence of a toxic substance in Experiment X. If such a substance exists it must be present in very small quantity, and B. elegans must be sensitive to very small changes in its concentration.

From the above it must be concluded that it is impossible to determine the exact pH range for B. elegans on gelatin media, since this depends on the concentration of the gelatin, the intensity and length of time of the sterilisation process, and probably other factors. Thus the discrepancies noted in Experiments VIIa and VIIb can be accounted for, not only by the different times of sterilisation employed (see Table 14) but also by slight differences in the concentration of gelatin in media made up on two different occasions.

The experiments described in this section show clearly that it is not possible to draw sound conclusions from results

obtained with fungi grown on gelatin media, for it seems very improbable that B. elegans is the only fungus which is so highly sensitive to slight differences in the composition of gelatin media. Since gelatin media were found to be so unsuitable for physiological experiments it was decided to determine the pH range for B. elegans on silica gels.

SECTION 5. THE INFLUENCE OF pH VALUE ON GROWTH IN SILICA GEL MEDIA.

Styer (1930 a) published details of a method for making silica gels using potassium silicate and phosphoric acid. This method with certain modifications was employed with great success in this investigation.

A potassium silicate solution was used containing 8% SiO_2 (molecular ratio K_2O: SiO_2 = 1.36) and a 0.2M phosphoric acid solution in which the nutrients were dissolved. The solutions were made up with air free distilled water; this precaution ensures that air-bubbles do not form in the gel during autoclaving. By means of an automatic pipette 10 ccs of medium was poured into each petri-dish and left to set for at least six hours. A given pH value was obtained by leaving 10ccs of a citric acid solution of the correct concentration, as previously determined by experiment, in contact with a silica gel plate for 6-12 hours to attain equilibrium; after determining the pH value, the supernatant liquid was poured away. The media were sterilised by autoclaving for 20 mins at 15 lbs pressure.

Experiment XI This was performed to determine the relation between pH value and growth for B. elegans. The silica media had the following composition after dialysis, Table 18.

Table 18. The Composition of the Media in Experiment XI.

pH Value	Glucose	NH_4NO_3	$MgSO_4 .7H_2O$	Pot. phosphates	Citric acid	SiO_2 (hydrated)
3.2-3.4	0.1 M	0.004 M	0.0004 M	0.037 M	0.037 M	2% approx.
3.8	"	"	"	"	0.027 M	"
4.0	"	"	"	"	0.025 M	"
4.4	"	"	"	"	0.022 M	"
4.8	"	"	"	"	0.017 M	"
5.4	"	"	"	"	0.012 M	"
5.6-5.8	"	"	"	"	0.01 M	"
6.2-6.4	"	"	"	"	0.007 M	"
6.6	"	"	"	"	0.005 M	"

The media were inoculated from two oat agar cultures 21 days old. The pH values of the media at the margin of the cultures were determined at intervals during growth. In all cultures it was found that the media became increasingly acid during growth so that the effect of any particular pH value on the rate of growth could only be determined during the early part of the experiment. After 7-9 days this alteration of the pH values had become very appreciable in the media commencing at pH 5.4, pH 5.6-5.8, and pH 6.2; but it was less marked in media starting at pH 3.8, pH 4.0, pH 4.4 and pH 4.8.

Table 19. The Average Diameters in Cms of Cultures in Experiment XI, Fig. 19.

No. of days after inoculation		2	5	7	9	12	14
pH value of medium	No. of cultures averaged						
3.2-3.4	4	0.35	0.35	0.35	0.35	0.35	0.35
3.8	7	0.37	0.6	0.77	0.93	1.19	1.28
4.0	9	0.36	0.59	0.77	0.89	1.05	1.17
4.4	9	0.38	0.59	0.81	0.98	1.2	1.3
4.8	9	0.4	0.55	0.76	0.98	1.17	1.21
5.4	9	0.37	0.44	0.5	0.59	0.77	0.86
5.6-5.8	9	0.36	0.39	0.41	0.52	0.62	0.76
6.2-6.4	5	0.35	0.35	0.35	0.4	0.5	0.53
6.6	2	0.35	0.35	0.35	0.35	0.35	0.35

Table 20. The increase in Diameter 9 days after inoculation. Experiment XI, Fig. 20.

pH values	3.2-3.4	3.8	4.0	4.4	4.8	5.4	5.6-5.8	6.2-6.4	6.6
Diameter in cms	0	0.58	0.54	0.63	0.63	0.24	0.17	0.05	0

Figures 18 (a-g) show the growth curves of the individual cultures on the various media; growth was uniform within each set. The average growth curves of the cultures at each pH value are shown in Figure 19, where a sudden rise in the steepness of the curves is to be noted about the 7th day on media F, G and H due to the more vigorous growth induced by the increased acidity. Therefore in a comparison of the effect of the different media upon growth it was decided to use the average increase in diameter 9 days after inoculation, Figure 20, page 59a.

The range of pH value possible on this medium covers ~~the~~ values from 3.2-3.4 to 6.4. The region of optimum growth lies between 3.8-4.8.

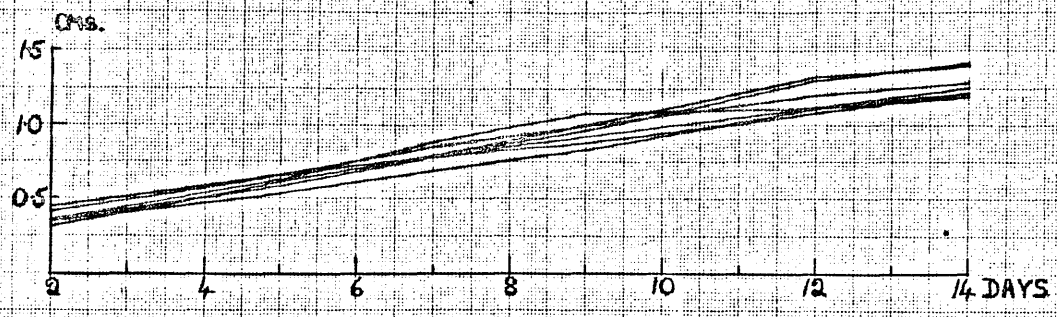
It has been noted in connection with the gelatin experiments in Section 4 that B. elegans has a different pH range on the solid and liquid forms of the same medium (see Experiment IX). It seemed advisable therefore to test if this is also the case in relation to silica gels.

Experiment XII. 100 ccs flasks were used, each containing 20 ccs of medium of the following compositions, see Table 21, corresponding to the nutritive media used in the silica gel cultures (cf. Table 18).

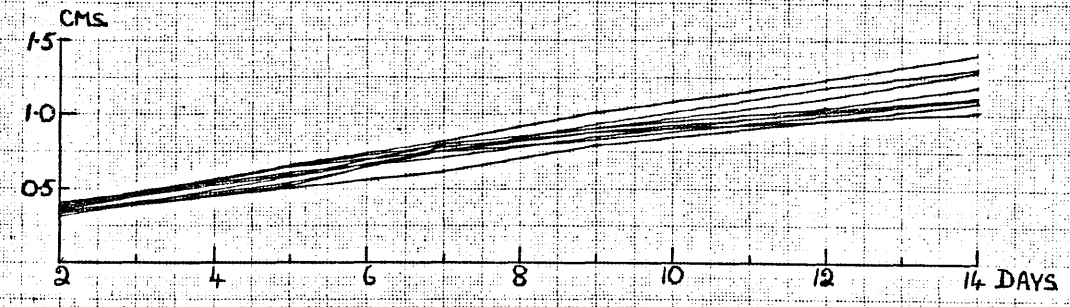
FIG. 18. THE GROWTH OF CULTURES ON SILICA GEL MEDIA.

EXPT. XI.

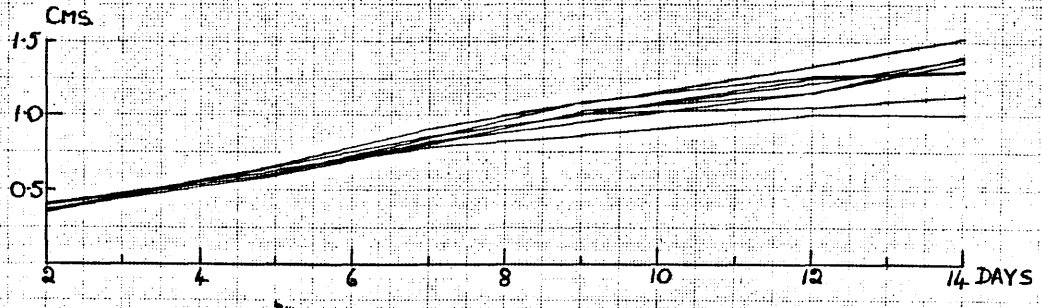
(a) pH VALUE 3·8 → 3·6



(b) pH VALUE 4·0 → (3·6–3·8)



(c) pH VALUE 4·4 → (3·8–4·0)



(d) pH VALUE 4·8 → 4·2

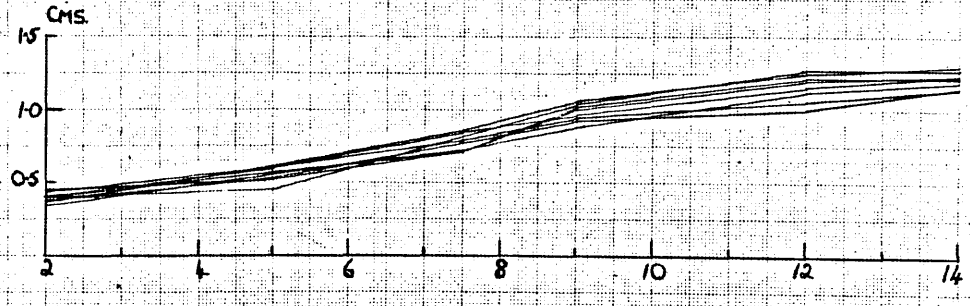
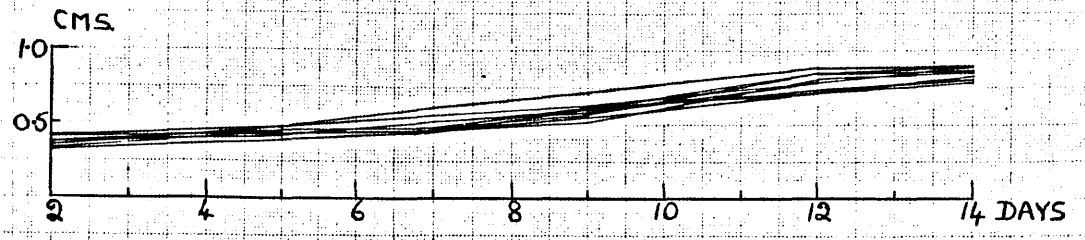
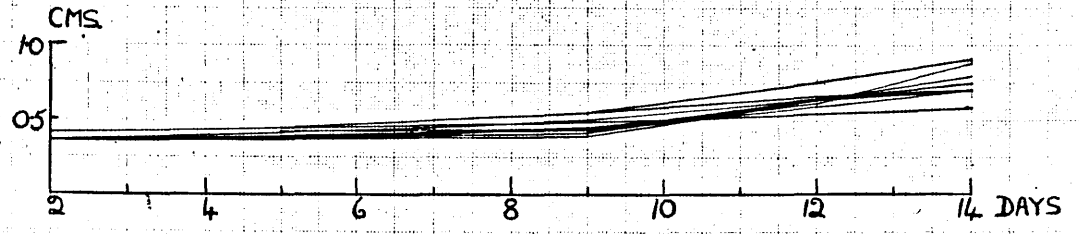


FIG. 18. (CONTINUED)

(e) pH VALUE 5·4 → 4·8



(f) pH VALUE (5·8 – 5·6) → 5·2



(g) pH VALUE (6·4 – 6·2) → 5·4

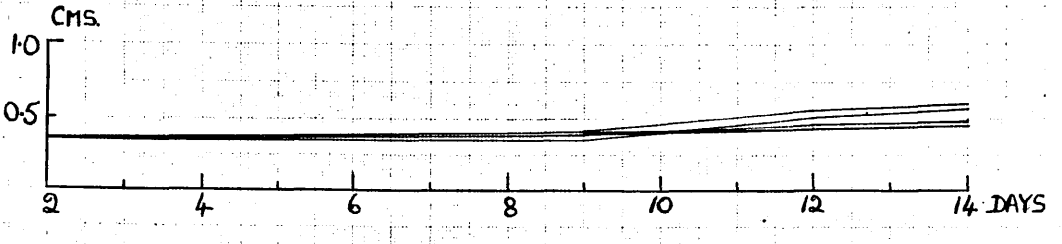


FIG. 19. COMPARISON OF GROWTH ON SILICA GEL MEDIA. EXPT. XI

TABLE 19.

A. pH (3·2 – 3·4)	D. pH 4·4	G. pH 5·6 – 5·8
B. pH 3·8	E. pH 4·8	H. pH 6·2 – 6·4
C. pH 4·0	F. pH 5·4	J. pH 6·6

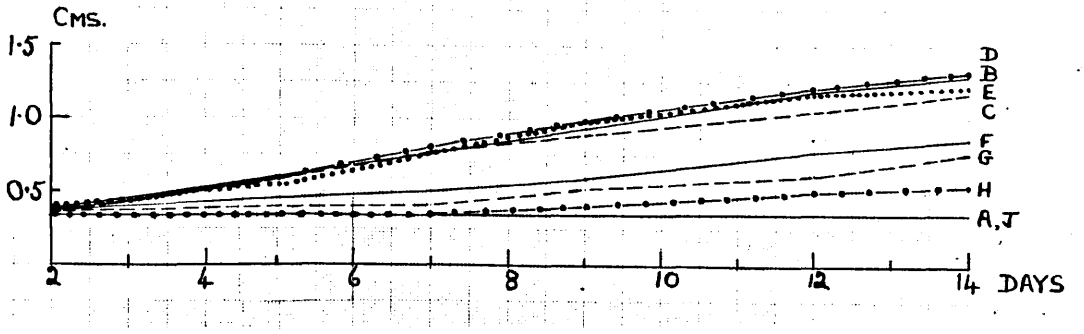


Table 21. The Composition of the Media in Experiment XII.

pH value	Glucose	NH_4NO_3	$MgSO_4 .7H_2O$	Pot. phosphates	Citric acid
3.6-3.8	0.1 M	0.004 M	0.0004 M	0.037 M	0.041 M
4.4	"	"	"	"	0.034 M
5.4	"	"	"	"	0.025 M
6.0-6.2	"	"	"	"	0.017 M
6.6-6.8	"	"	"	"	0.01 M

The flasks were inoculated from a beerwort culture 23 days old. Since only the growth reaction of B. elegans to these media was required, the dry weights of the cultures were not determined. The effect of the media on the growth of the inocula is shown in Table 22.

Table 22. Growth Reaction of Cultures in Experiment XII

pH value	5 days after inoculation	9 days after inoculation	33 days after inoculation
3.6-3.8	No growth	Very poor growth	Very poor growth
4.4	Vigorous growth	Vigorous growth	Very vigorous growth
5.4	Very poor growth	Poor growth	Fairly vigorous growth
6.0-6.2	No growth	No growth	Very poor growth
6.6-6.8	"	"	No growth

It is evident that the range of pH value possible for growth in this liquid medium extends from pH 3.6-3.8 to 6.0-6.2. This is only slightly less extended than that obtained on the corresponding silica medium, viz. 3.2-3.4 to 6.4. It is easy to account for this difference in the fact that liquid media do not usually give such good growth as solid media and hence in the former the range of pH value possible for growth will be shortened at both ends. The important conclusion is thus reached that results obtained on silica gel media can be taken as a true representation of the growth of B. elegans in pure culture on any particular set of nutrients, whereas results obtained on gelatin media cannot.

Although the source of nitrogen in this experiment was NH_4NO_3, there seems no doubt that only the ammonium was utilised as shown by the increasing acidity of the medium (see Experiment II, Section 3). However, in the absence of ammonium, nitrate can be used as a nitrogen source for B. elegans (see Section 3) and the range of pH value in that case was determined in the next experiment.

Experiment XIII. The silica media had the following composition, see Table 23.

Table 23. The Composition of the Media in Experiment XIII

pH value	Glucose	KNO_3	$MgSO_4 .7H_2O$	Pot phosphates	Citric acid	SiO_2 hydrated
3.2-3.4	0.1 M	.004 M	0.0004 M	.037 M	0.045 M	2% approx.
3.6	"	"	"	"	0.035 M	"
4.4	"	"	"	"	0.025 M	"
5.0	"	"	"	"	0.017 M	"
5.4-5.6	"	"	"	"	0.014 M	"
6.2	"	"	"	"	0.01 M	"
6.8	"	"	"	"	0.006 M	"

Table 24. The increase in Diameter 15 days after inoculation. Experiment XIII. Fig.21.

pH values	3.2	3.4	3.6	4.4	5.0	5.4-5.6	6.2
No. of cultures averaged	2	6	8	7	11	11	11
Diameter in cms	0.21	0.45	0.57	0.8	0.37	0.11	0

The media were inoculated from 3 oat agar cultures 23 days old. There was no change in the pH value of the media during the period of the experiment.

Since nitrate is not a good source of nitrogen for B. elegans the growth of all cultures was poor. The average

diameters obtained after 15 days growth are given in Table 24 and shown graphically in Figure 21. Unfortunately there were no cultures with a pH value below 3.2; however pH 3.2 can only be just above the minimum value, since growth on this acidity was extremely slow.

The pH range on the nitrate medium is therefore 3.0-3.2 to 5.6-5.2. The range is somewhat shorter towards the alkaline end than on the corresponding ammonium medium. While this difference is doubtless a reflection of the poorer nitritive qualities of the nitrate medium, it may also be due to the tendency to increasing alkalinity shown in nitrate cultures. Growth in an ammonium medium of pH 6.2 early results in an increase in acidity beneficial to growth; in a nitrate medium no such beneficial change can occur, and thus although germination of the inoculum is possible, little growth takes place. At the acid end of the pH scale, however, growth on the nitrate medium would produce a beneficial deflection towards alkalinity and thus growth would be possible at a greater acidity than in an ammonium medium. There is some indication of this, since the inocula germinated on pH 3.2 on the nitrate medium but failed to do so on pH 3.2-3.4 on the ammonium medium.

The general shape of the curves in Figures 20 and 21 are similar - optimum occurring in the region of pH 4.6. From the two silica gel experiments one concludes that the maximum range

FIG. 20. GROWTH IN RELATION TO pH VALUE ON SILICA GELS. EXPERIMENT XI

(NH_4NO_3 MEDIUM.) TABLE 20.

——— AVERAGE DIAMETERS 9 DAYS AFTER INOCULATION.

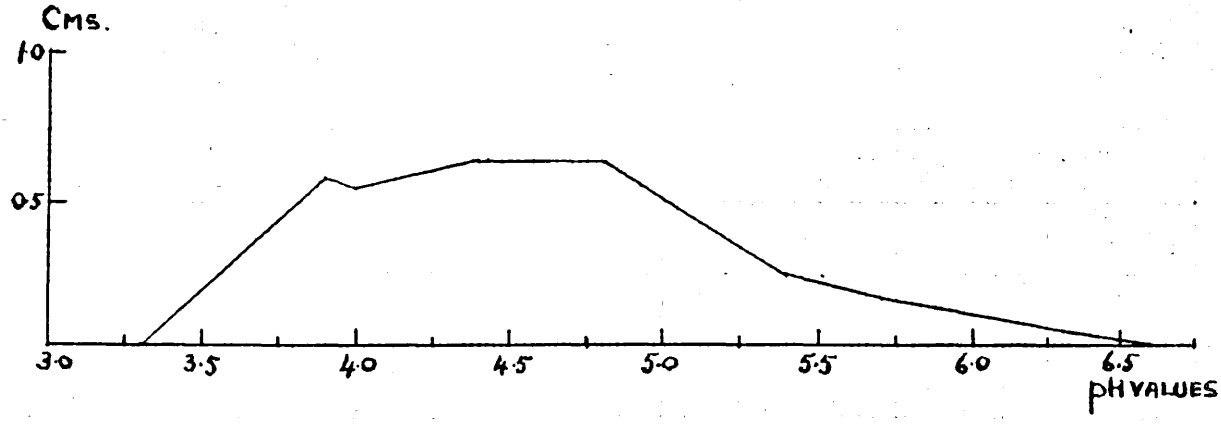
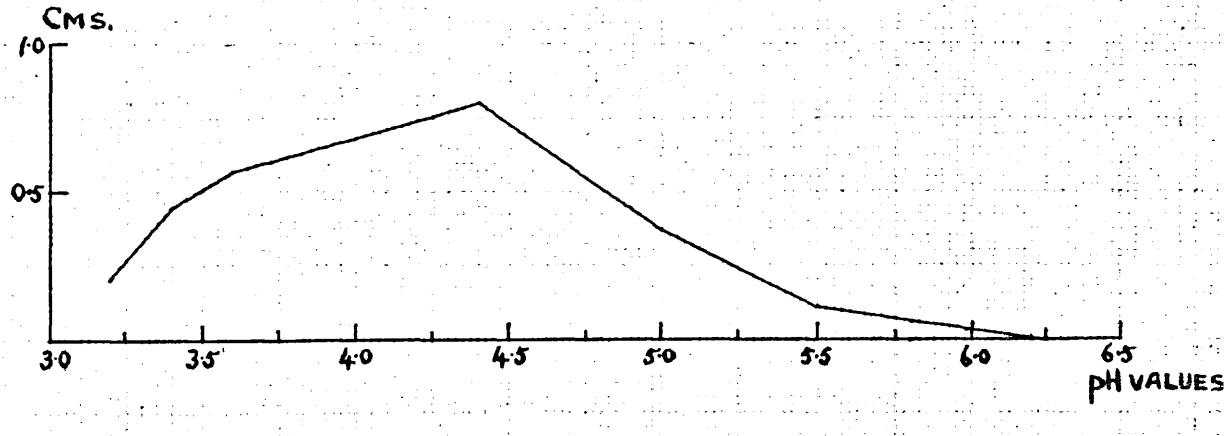


FIG. 21. GROWTH IN RELATION TO pH VALUE ON SILICA GELS. EXPERIMENT XIII

(KNO_3 MEDIUM). TABLE 24.

——— AVERAGE DIAMETERS 15 DAYS AFTER INOCULATION.



of pH value possible for growth in media containing glucose, salts and inorganic nitrogen compounds is pH 3.0-3.2 to pH 6.4.

SECTION 6. THE VALUE OF CERTAIN NITROGEN SOURCES FOR GROWTH

A thorough investigation into the sources of nitrogen possible for the growth of B. elegans has not been undertaken. One experiment was performed to determine the relative values of NH_4CL, KNO_3 and asparagine.

Experiment XIV

Silica gel media were used of the following composition:

Glucose	0.1 M
$MgSO_4.7H_2O$	0.004 M
Pot.phosphates	0.037 M
Citric acid	0.02 M
SiO_2 (hydrated)	2% approx

To this were added solutions of NH_4Cl, KNO_3 and asparagine so that the ~~fixed~~ final concentration of nitrogen was 0.05 M. The pH value was 4.4-4.6 in all media. The petri dishes were inoculated from 2 oat agar cultures 25 days old.

Growth on the ammonium medium was regular, uniform and almost entirely on the surface of the substrate. On the nitrate medium, however, the colonies were irregular in shape and very variable in size; moreover a considerable amount of growth occurred below the surface of the substrate. On the asparagine medium growth, though slow, was very uniform. The average diameters obtained from 11 cultures on each medium are shown in Table 25, and in Figure 22.

FIG. 22. THE RELATION OF GROWTH TO NITROGEN SOURCE. EXPERIMENT XIV

TABLE 25.

---- AVERAGE DIAMETER ON NH_4Cl.

•——• " " " KNO_3.

——— " " " ASPARAGINE.

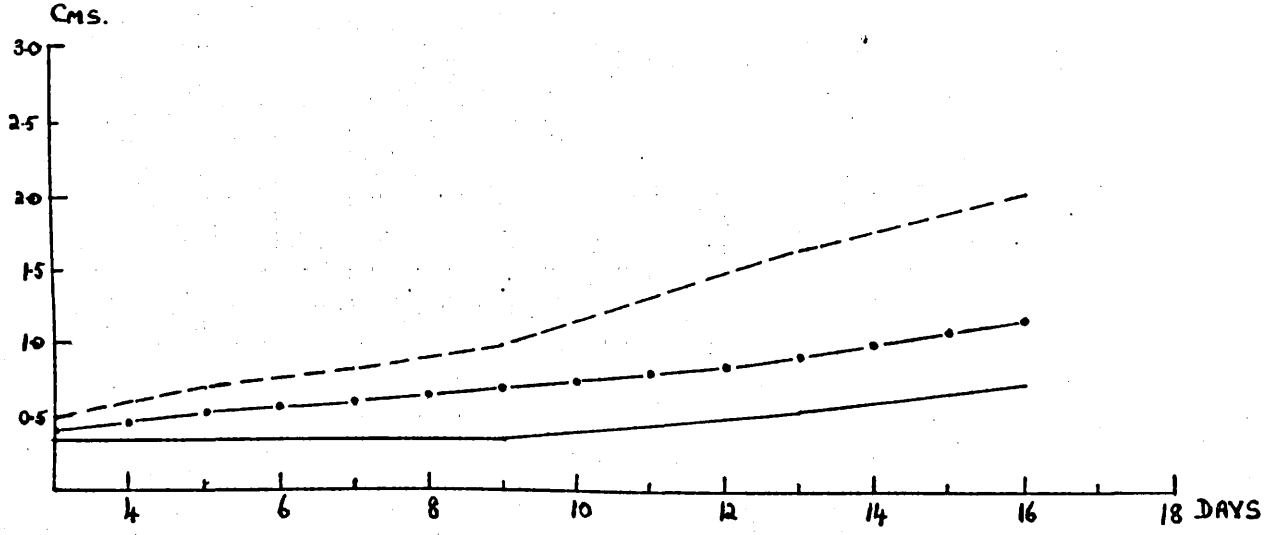


Table 25. Average Diameters in Experiment XIV Fig.22.

No. of days after inoculation	NH_4Cl	KNO_3	Asparagine
	cms	cms	cms
3	0.49	0.41	0.34
5	0.69	0.51	0.34
7	0.82	0.57	0.34
9	0.99	0.69	0.35
13	1.62	0.9	0.53
16	2.0	1.15	0.71

There is no doubt that B. elegans in pure culture tends to be an ammonium organism, with only a limited ability for using nitrate. This conclusion is further borne out by the results from Experiment II, Section 3 when NH_4NO_3 was used as a source of nitrogen. The rapid increase in acidity indicates that although both ammonium and nitrate were present, only the ammonium was utilised.

Melin (1925) noted that B. elegans could liquefy gelatin; this has been confirmed (see Experiment IV, Section 3). In this investigation slow growth was obtained on 0.5% peptone in silica gel media.

Melin (1925) concluded that all true mycorrhizic fungi were limited to the ammonium salts of inorganic acids and

certain protein compounds for their supply of nitrogen. While B. elegans can use nitrate, asparagine, peptone and gelatin, nevertheless it appears to be a typical mycorrhizic fungus, growing most vigorously in the presence of ammonium compounds.

SECTION 7. THE INFLUENCE OF MOLECULAR CONCENTRATION.

Styer (1930b) in his work on the mushroom found that the molecular concentration of the nutritive solution influenced the rate of growth; growth was retarded in solutions above 0.2M. No experiments have been carried out with the express object of determining the effect of molecular concentration on the growth of B. elegans. Certain facts, however, have come to light which suggest that for this fungus there is an upper limit of molecular concentration beyond which growth is affected adversely. Where the upper limit lies is as yet undetermined.

The three following experiments show the influence of molecular concentration.

Experiment XV. The liquid culture medium consisted of K_2SO_4, NH_4Cl and a phosphate-citric buffer solution to which four different concentrations of glucose were added, 1.5%, 2.0%, 2.5% and 3.0%. The pH value of each solution was 4.6 after autoclaving for 20 mins at 15 lbs pressure. The media were inoculated from a beerwort agar culture 25 days old. The molecular concentrations are given in Table 26.

Table 26. Molecular Concentrations of Media in Experiment XV.

Solution	Nutrient Solution	Phosphate buffer	Citric acid buffer	Glucose	Total Concentration	Growth reaction
A	0.02 M	0.07 M	0.04 M	0.083 M	0.213 M	xxx
B	"	"	"	0.111 M	0.241 M	xx
C	"	"	"	0.139 M	0.269 M	xx
D	"	"	"	0.167 M	0.297 M	xx

The cultures were grown in test tubes each containing 5ccs of media. The colonies were not measured but the amount of growth is represented in Table 26, where xxx indicates that the inocula showed rapid germination and vigorous growth and xx that the inocula were slower in germinating and in subsequent growth attained between $\frac{1}{2}$ and $\frac{4}{5}$ size of xxx cultures. The only reasonable explanation of the difference between solution A and the other three solutions is that the increased supply of energy available for growth in solutions B, C and D could not be utilised owing to the adverse molecular concentration of the nutrients.

Experiment XVI. Two gelatin media were sterilised by steaming for 20 mins on 3 successive days, and were inoculated from two oat agar cultures 43 days old. The concentrations of nutrients is compared in Table 27.

Table 27. Composition of Media in Experiment XVI.

Nutrients	Medium E	Medium F
Glucose	0.1M	0.1M
* $MgSO_4 .7H_2O$	0.0004 M	-
K_2SO_4	-	0.0006 M
KH_2PO_4	0.007 M	-
Na_2HPO_4	-	0.087 M
NH_4Cl	0.009 M	0.018 M
Citric Acid	-	0.031 M
Total molecular concentration	0.1164 M	0.2366 M
Gelatin	10%	10%
pH value after sterilisation	5.2-5.4	5.4

* It was found by experiment that Mg ions exert no appreciable effect upon growth.

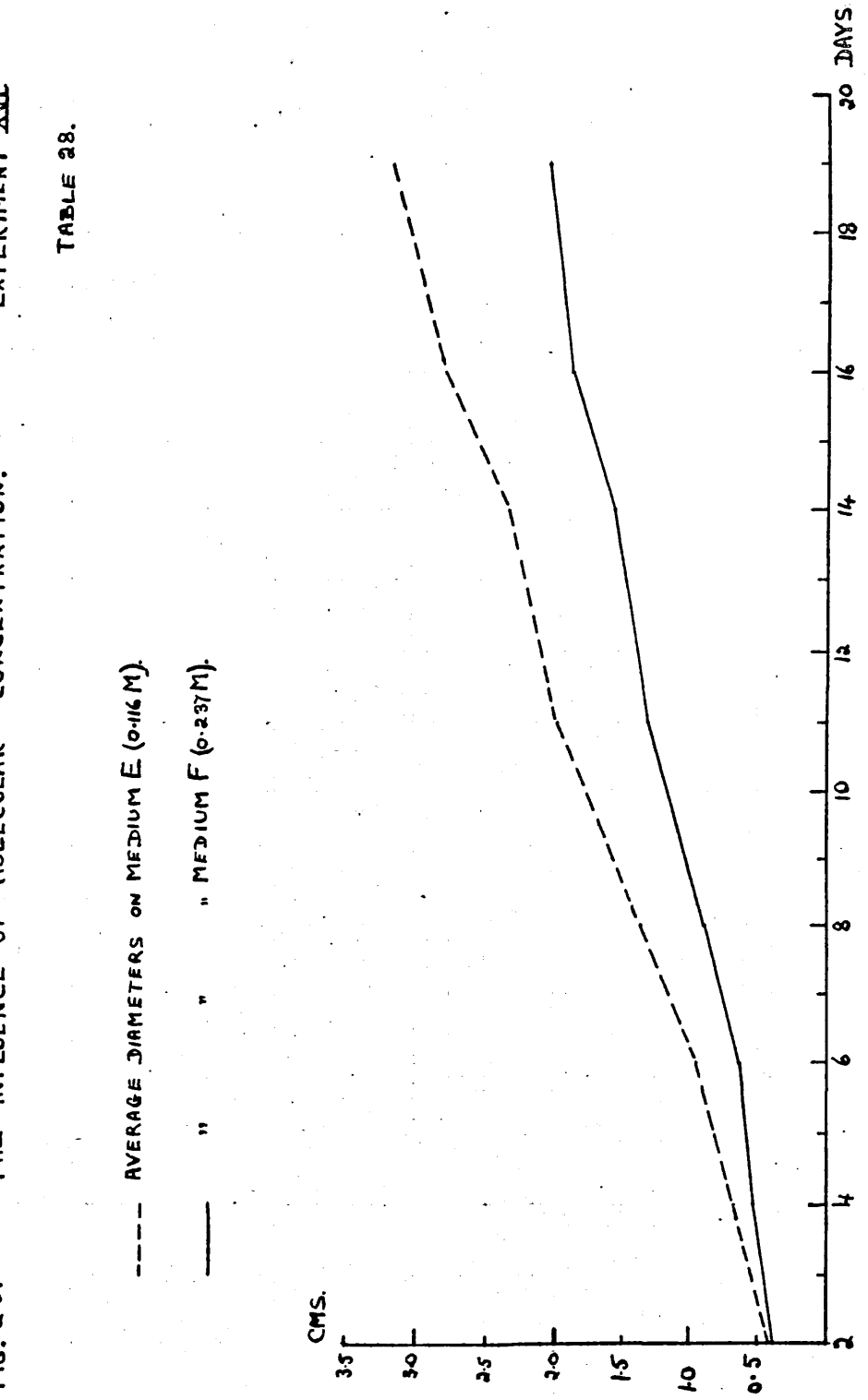
Table 28. Average Diameters of Cultures in Experiment XVI Fig. 23.

No. of days after inoculation	2	4	6	8	11	14	16	19
Average Diam. in cms of 9 cultures on Medium E.	0.42	0.67	0.95	1.35	1.96	2.3	2.75	3.11
Average Diam. in cms of 8 cultures on Medium F.	0.39	0.53	0.62	0.88	1.29	1.52	1.83	2.0

Table 28 and Figure 23 show the average diameters obtained on the two media. Although medium F contains considerably more

FIG. 23. THE INFLUENCE OF MOLECULAR CONCENTRATION. EXPERIMENT XXI

TABLE 28.



nitrogen and phosphate than medium E, growth on F was very much poorer than on E. Again it is suggested that the poorer growth is due to the deleterious effect of high molecular concentration.

Experiment XVII. Two solutions were employed for the experimental media.

1. Buffer Solution.

0.2 M Na_2HPO_4 solution	300.16 ccs
0.1 M citric acid solution	259.84 ccs

2. Nutrient Solution

Glucose	22.5 gms
K_2SO_4	0.125 gms
NH_4Cl	1.25 gms

Three 10% gelatin media were made containing:-

I	Nutrient solution	+	100%	buffer solution	
II	" "	+	75%	"	"
III	" "	+	50%	"	"

The media were autoclaved for 20 mins at 15 lbs and inoculated from 2 oat agar cultures 38 days old. The final composition in terms of molecular concentration is shown in Table 29.

Table 29. Molecular concentrations of Media in Experiment XVII.

Medium	Nutrients	Phosphates	Citric acid	Total Mol. conc.	pH after sterilisation
I	0.12 M	0.086 M	0.037 M	0.243 M	5.0
II	0.12 M	0.064 M	0.028 M	0.212 M	5.0
III	0.12 M	0.043 M	0.018 M	0.181 M	5.0

The results are shown in Table 30 and in Figure 24, from which it appears that medium III, although the poorest in nutrients, produces the best growth. This experiment was repeated with similar results.

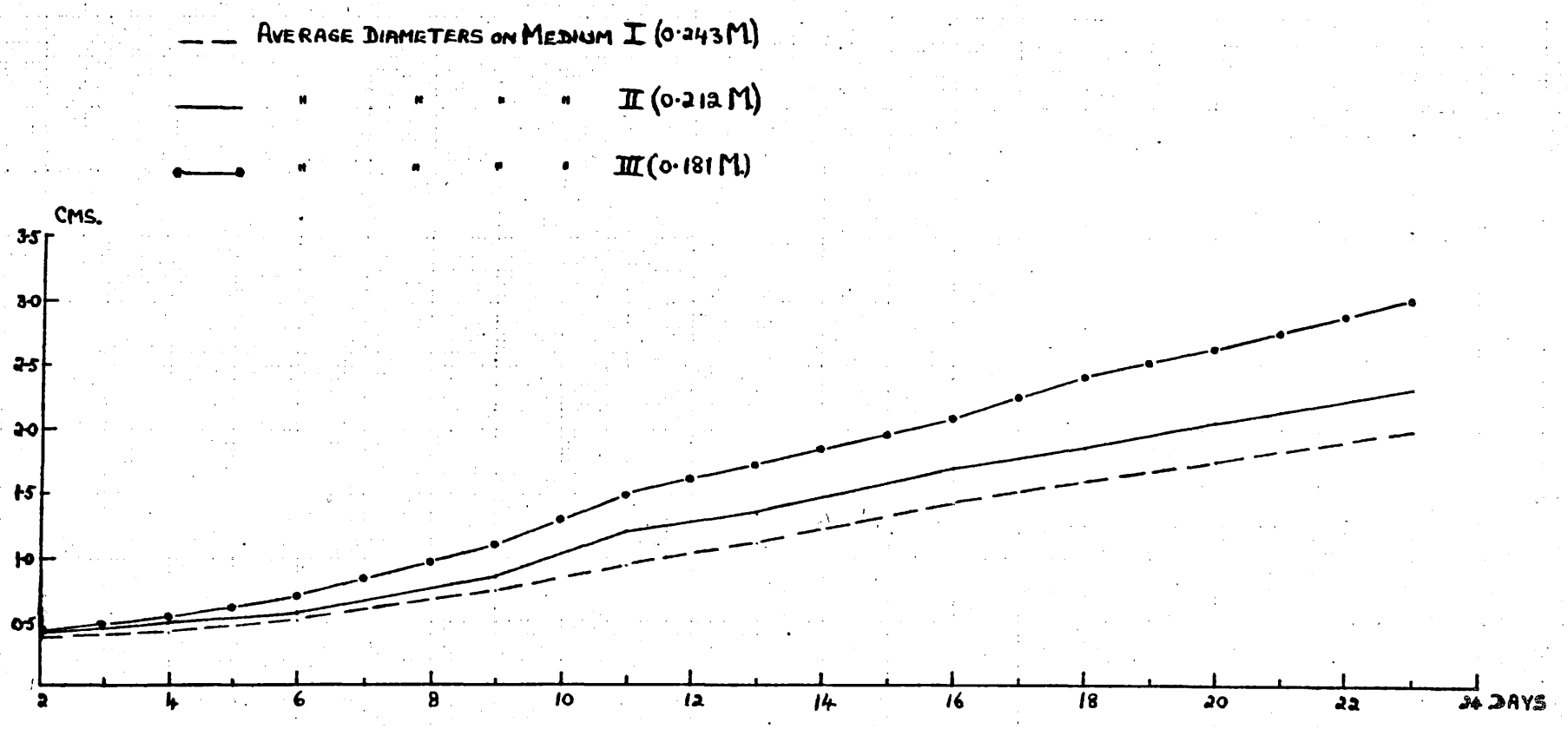
These three experiments XV, XVI & XVII, leave little doubt that there is a limiting value for the molecular concentration of the nutrient solution, above which the growth is adversely affected. It is possible that this value lies between 0.18 M and 0.21 M, when it would be similar to that found by Styer for the mushroom, but further experiments are necessary to determine this value exactly. It is clear, however, that the molecular concentration of nutrient media exerts an influence on growth which must be considered when drawing conclusions from experimental results.

Table 30. The Average Diameters of Cultures in Experiment XVII. Fig. 24.

No. of days after inoculation	Average Diameters in Cms.of 10 Cultures		
	Medium I	Medium II	Medium III
2	0.37	0.4	0.4
4	0.43	0.49	0.53
6	0.51	0.56	0.7
9	0.74	0.85	1.1
11	0.94	1.2	1.49
13	1.11	1.33	1.72
16	1.42	1.69	2.08
18	1.59	1.86	2.42
20	1.74	2.05	2.63
23	1.99	2.32	3.02

FIG. 24. THE INFLUENCE OF MOLECULAR CONCENTRATION. EXPERIMENT XVII.

TABLE 30.



DISCUSSION

The present investigation has been concerned only with the mycelium of B. elegans in pure culture; that is to say with a mycelium which had grown out from a sporophore - presumably diploid - on to a sterile nutrient medium, from which it was subcultured on to various substrates at frequent intervals. It is not known how closely the mycelium obtained from such a tissue culture resembles in morphology the natural mycelium of the fungus, nor to what extent the physiological characteristics determined in these artificial conditions give a true picture of the behaviour of the fungus. It is important to bear this in mind when drawing conclusions from results obtained in pure culture work, particularly when the fungus concerned cannot be grown from spores and hence the 'tissue culture' mycelium cannot be compared with one more natural in origin. B. elegans is such a fungus and the following discussion of its mycelial characters applies only to the 'tissue culture' mycelium.

Certain features such as 'paarige' branching, papillose ~~ex~~se cretions, etc. have been found in the mycelia of all Boletus spp. so far grown in culture. It is probable that these and similar features are of generic value, a view which is borne out by a comparison with the mycelium of other genera, e.g. Tricholoma terreum. A comparison of the mycelia of the Swedish

and English strains of B. elegans, however, raises the question as to how far the mycelia of Boletus spp. show specific differences in culture. Before considering this, it is necessary to decide what part the 'vigour' of the fungus plays in determining the mycelial characters. In this investigation 'vigour' is used to describe the relative amount and quality of growth of a culture, so that a vigorous culture shows a high rate of growth and produces a dense aerial mycelium. It is possible that some cultures of a fungus are inherently more vigorous than others, but it appears more probable that for any particular fungus 'vigour' depends upon environmental factors. It is important to note that these factors include not only the growth conditions of the fungus at the particular time in question, but also the growth conditions of previous mother cultures (Section 2). The evidence does not suggest that the influence of this last factor works through changes in the inherent character of the fungus occurring during growth, but rather through the presence of staling products in the inocula, the quality and intensity of which depend on previous conditions. There is the further possibility that the physiological condition of the sporophore at the time of isolation may affect the 'vigour' of the particular strain, an effect which may disappear during cultivation. Certainly in this investigation there is little

evidence that vigorous growth is the result of any factors other than environmental conditions.

From Melin's work it is possible to distinguish two types of mycelium within the genus Boletus; one in which 'paarige' branching and clamp connections are frequent, the other in which they are very infrequent or altogether absent. At first sight it appears that these two types are fundamentally distinct. However, it has already been noted (Part I) that these particular mycelial characters vary in frequency in B. elegans with the vigour of growth. Thus the Swedish strain which grew less vigorously than the English strain shows the mycelial characteristics of the second type rather than the first, while the English strain approximates more closely to the first type. It is therefore possible that all Boletus spp. when growing vigorously in culture produce mycelia of the first type, but that certain species have so far only exhibited poor growth in culture and hence the second type of mycelium. In this latter case the environmental conditions do not permit the full expression of the mycelial characters.

Since, however, the sporophores of the various species have definite taxonomic characters it seems unlikely that the mycelia derived from them would exhibit no inherent differences. But with so many unknown environmental factors, including the condition of the sporophore at the time of isolation, it does not

seem possible to control conditions so that all cultures will grow equally vigorously and show fixed mycelial characteristics. It is therefore not possible to control the limits between which the mycelium of any one species can vary, and hence it is not possible to ascribe distinct mycelial characters to the various species. One can only conclude that the Boletus spp. so far known in culture show certain generic mycelial characters, and that the extent to which these occur in the various species depends in great measure on the cultural conditions.

It has been reported that many Boletus spp. are difficult to keep in culture (Melin 1925). While this is scarcely true for B. elegans, since little difficulty was experienced in keeping stock cultures vigorous, undoubtedly the sensitiveness of the fungus to slight changes in cultural conditions renders it a difficult subject for physiological study; this is particularly so with regard to its sensitiveness to the metabolic products of its own growth. The intolerance of B. elegans to staling products combined with the necessity of using hyphal inocula for propagation, since no accessory spores are formed, results in great irregularities in the vigour and size of cultures. This consideration makes it essential if trustworthy conclusions are to be drawn from experiments to use only the average results of cultures identical in past history.

This method has been employed wherever possible in the present investigation, since it is the only way to obtain results comparable in value to those derived from fungi producing accessory spores. With the latter organisms no difficulties arise in respect either of the staling products of the mother cultures, or the differing amounts of hyphae in the inocula, since it is possible to use monosporous cultures.

During the course of this enquiry it became apparent that the molecular concentration of the substrate is a factor which may sometimes be of importance in determining growth. Very little attention seems to have been paid to this aspect of culture media. In B. elegans, as in the Mushroom, the influence of molecular concentration is apparent in the region of .2M and above (see Section 7). While slight differences in molecular concentration probably do not affect growth, big differences may entirely mask the influence of other factors; it is therefore essential to consider the molecular concentration when interpreting results. If this phenomenon is an expression of the influence of osmotic pressure upon growth, it should be of general occurrence and investigations into the effect of molecular concentration should reveal a similar condition for other fungi. The results obtained with B. elegans show that molecular concentration of the substrate is often the controlling factor, overriding other factors, such as the increased nutritional value

of the substrate, and is of such importance that it cannot be ignored.

There is little evidence that B. elegans differs greatly from many other fungi in the physiological characters dealt with in these experiments. Harley (1934) has shown that in certain moulds the source of nitrogen determines the direction of the deflection in pH value occurring during growth. B. elegans behaves similarly, producing an increased acidity in the substrate when the source of nitrogen is an ammonium compound and an increased alkalinity when it is a nitrate, or when excess ammonia is present in gelatin cultures (see Section 3).

Although B. elegans shows a decided preference for ammonium as a source of nitrogen, it can also use gelatin, peptone, asparagine and nitrate, (see Section 6). If conditions in pure culture are at all comparable to those in nature, B. elegans will derive its nitrogen in the soil from ammonium sources when these exist, and from various protein derivatives. Melin (1925) found that all the mycorrhizic fungi he examined used ammonium and urea easily, but the different species varied considerably in their ability to use other nitrogen sources; on the whole nitrates were a poor source of nitrogen, and gelatin, legumin, peptone and asparagine were only of value to certain species. The evidence in the present enquiry indicates that in

regard to its nitrogen diet B. elegans differs in no fundamental respect from the other mycorrhizic fungi investigated.

The chief concern of the present investigation, however, was the growth relation of B. elegans to acidity. Many coniferous woodland soils are markedly acid - in the region of pH 5.0 - and in such conditions the mycorrhizic Boleti exist. It is therefore to be expected that the pH range for these fungi will be on the acid side of neutrality. Melin (1925) showed that this was the case for Boletus luteus, B. granulatus, B.variegatus and B. badius. The pH range in 5% malt extract extended from below pH 3.3 to above pH 7.2, with optimum growth in the region of pH 5, except in the case of B. granulatus which showed optimum development at pH 5.7-6.0. The greatest pH range which has been obtained for B. elegans occurred on silica gel media with ammonium as the source of nitrogen; in these conditions growth occurred between pH 3.2-3.4 and 6.4 (section 5). In the corresponding liquid media the range was less, extending from pH 3.6-3.8 to 6.0-6.2. B. elegans has therefore a distinctly shorter range than the species of Boletus investigated by Melin, growth becoming impossible at the pH values near the neutral region of the scale. The optimum values in the silica gel media lie between pH 3.8 and 4.8 on both NH_4NO_3 and KNO_3; compared therefore with other Boletus spp. optimum growth for B. elegans occurs in more acid media.

Now the silica gel media contained mineral salts, glucose and citric acid, no other organic compounds being present; also the results on the silica gels were very similar to those in liquid media of similar composition; therefore it can be inferred that in the absence of modifying factors B. elegans has a pH range of 3.2-3.4 to 6.4, with optimum growth in the region of pH 3.8-4.8. It is to be expected that on unfavourable media, this range will be shortened at both ends, but the optimum growth should still occur in the same region of pH 3.8-4.8. This is so in the case of liquid buffered media in which the molecular concentration is high; the pH range becomes reduced occurring between pH 3.6 and 5.6; the optimum, however, remains in the region of pH 3.8-4.6.

The significance of the gelatin experiments reported in Section 4 lies in the possibility that here modifying factors exist, which bring about a change in the pH range and a deflection of the region of optimum growth. It has been postulated that in Experiment X three factors combine to alter the position of the pH range. First a toxic substance is produced during the hydrolysis of the gelatin in acid solution, the concentration of which is greatest in the most acid solutions and increases with increasing concentration of gelatin. This substance is sufficiently concentrated in solutions of pH 4.0-4.4 to prohibit

growth, but in pH 4.6-4.8 it only retards growth; above pH 4.8 the concentration of toxic substance is not sufficient to influence the fungus, which then becomes affected by the pH value of the medium and the increased nutritional value of the substrate due to the presence of gelatin. Thus the pH range is deflected from between 3.8-4.0 to 5.2-5.4 in the absence of gelatin to between 4.8 and 5.6+ in 10% gelatin, the optimum in the first case occurring at about pH 4.4, in the second case at about pH 5.0.

The above explanation assumes that B. elegans is extremely sensitive to very small changes in the concentration of the toxic substance. However, it is possible that whilst differing concentrations of the toxic substance play a rôle in determining the growth reaction, a further factor also exists, namely a decrease in toxicity of the products of gelatin hydrolysis with decreasing acidity. Such a condition is already known for toxic acid dyes. Robbins (1936) showed that 1 in 1,000 eosin entirely inhibited growth of Gibberella saubinetii at pH 4.0, but at pH 4.3, 30% normal growth was obtained, and he sums up the conditions as follows:- "A given concentration of a dye may be lethal at one hydrogen ion concentration and entirely or almost harmless at another". It remains for further investigation to determine whether a condition such as this operates in gelatin media.

Gelatin is an extremely variable substance hence there

is little agreement between investigators about important properties such as the position of the iso-electric point. It follows that any one of a number of unknown physical or chemical factors may determine the phenomenon under discussion. Whatever this factor or combination of factors may be, one organism at least is very sensitive to it; it seems highly improbable that B. elegans is the only fungus so affected, but rather that it is in general more sensitive to cultural conditions than the moulds and therefore it reveals the existence of factors in gelatin media which have so far been overlooked. Whatever the full explanation of the behaviour of the gelatin cultures may be there is no doubt that gelatin is a singularly unsuitable medium to employ in culture experiments designed to show the comparative effects of different nutrients on growth, since such effects may be entirely masked by those resulting from the sterilisation of the gelatin.

The present investigation has failed to shed any further light on the relationship between B. elegans and Larix decidua. The fungus appears to be less specialised in its physiological characters than Melin had suggested, It is therefore doubtful how much information experiments with pure cultures could yield in this connection. It is concluded that B. elegans, in pure culture, does not differ greatly from other members of the genus, either in mycelial characters or in the aspects of physiological behaviour so far investigated.

SUMMARY

(1) The mycelium of *B. elegans* is described and compared with previous descriptions. There is no evidence that the mycelium differs in any important respect from that of other *Boletus spp.*

(2) The quantity and quality of the staling products present in inocula influence the rate of growth of the cultures derived from them.

(3) During growth the pH value of the medium changes; the direction of change depends on the source of nitrogen.

(4) In gelatin media the pH range within which growth occurs and the pH value for optimum growth depend on the concentration of gelatin and the length of time of sterilisation. It is suggested that toxic substances formed during the hydrolysis of gelatin are responsible for the results obtained.

(5) The maximum range of pH values possible for growth in media containing glucose, salts and inorganic nitrogen compounds lies between pH 3.0-3.2 and pH 6.4.

(6) While nitrate, asparagine, peptone, and gelatin can serve as nitrogen sources, vigorous growth is only obtained with inorganic ammonium salts.

(7) The molecular concentration of the nutrient media exerts an appreciable influence upon growth.

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