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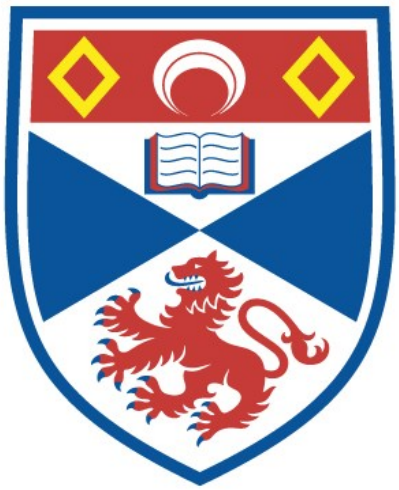

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CERTIFICATE

We certify that Mr. Drake Hocking has spent four terms of Research Work under our direction and that he has fulfilled the conditions of Ordinances No. 61 and 51 (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the degree of Master of Science in Pure Science.

DECLARATION

I hereby declare that this Thesis records work done by me, that it is a composition of my own, and that it has not been previously presented for a Higher Degree.

CAREER

I was graduated from the University of Alberta in May 1960, with the degree of B.Sc.(Arts), with First Class General Standing; when I was also awarded the Faculty of Arts and Science Gold Medal in the B.Sc. Program.

I was registered as a Research Student in the University of St. Andrews in October 1960 under Ordinances 61 and 51. The research was carried out in the Department of Botany at St. Salvator's College of the University of St. Andrews and in the Department of Botany at King's College of the University of Durham under the direction of Professor J.H.Burnett; and in the Gatty Marine Laboratory of the University of St. Andrews under the supervision of Dr. D.C.Weeks.

The period during which this research was carried out lasted from October 1960 to September 1961.

ACKNOWLEDGEMENTS

With grateful thanks, I acknowledge help and encouragement with the work from my supervisors, Professor J.H.Burnett and Dr. D.C.Weeks.

I also wish to thank Professor J.A.Macdonald for providing facilities in the Department of Botany at St. Andrews.

To my colleagues in the Departments of Botany of the Universities of St. Andrews and Durham, for their stimulating discussions, thanks.

For financial support during the period of study, I am grateful to the University of St. Andrews for a Post Graduate Scholarship; and to the National Research Council of Canada for a Special Scholarship; and to the British Council for a Travel Grant.

ASPECTS of CAROTENE ACCUMULATION by SOME MEMBERS of the MUCORACEAE

by Drake Hocking, B.Sc.(Arts), Alberta.

A Thesis submitted to the University of St. Andrews for the Degree of Master of Science.

Department of Botany,
King's College, University of Durham,
Newcastle upon Tyne, 1.

September, 1961.

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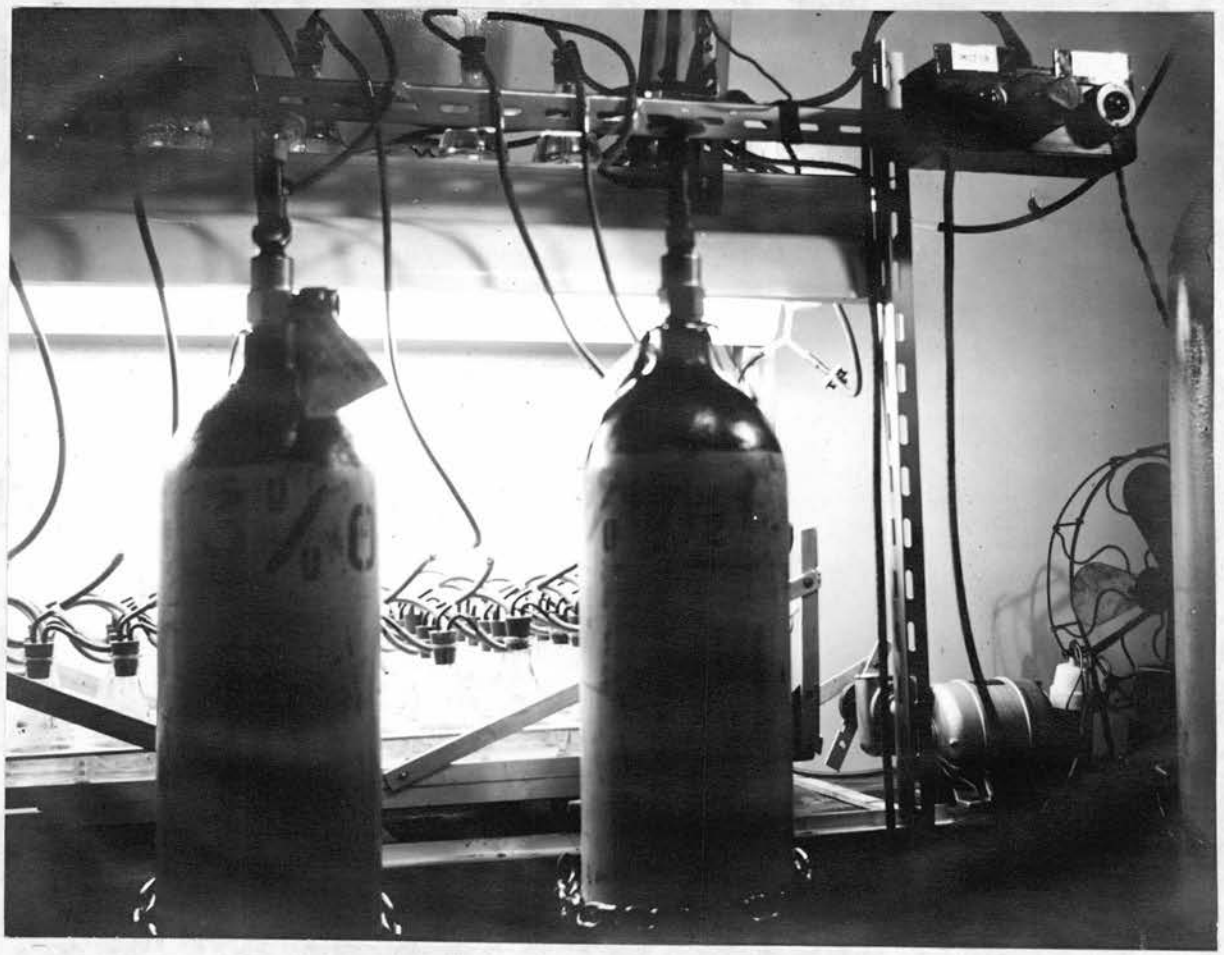
F R O N T I S P I E C E

The horizontal reciprocating shaking apparatus, as set up for an experiment with controlled oxygen tensions. In the foreground are cylinders of prepared gas mixtures. To the right may be seen the driving motor and heat-dissipating fan. Culture flasks are in the centre.



CONTENTS

Chapter I

INTRODUCTION & SURVEY OF PROBLEMS

INTRODUCTION & SURVEY OF PROBLEMS

Since first noticed, the bright yellow, orange and red pigments termed carotenoids have interested and puzzled investigators. Early workers, as Wolf(1917), simply report the appearance of "numerous yellowish oil globules" in fungal mycelium and immature zygospores. Later works on the occurence, formation and function of these intriguing substances have appeared at an autocatalytic rate.

Elegant biochemical techniques have revealed many separate but related compounds. Much activity in a search for new carotenoids has disclosed their extremely wide distribution. They occur in all principal groups of living organisms; yet within these groups there are anomolous species in which carotenoids have never been found. Biochemists yet search for a rationale.

In the Plant Kingdom, carotenoids appear in association with chlorophyls in photosynthetic tissues, and also in flower petals (Goodwin 1950). Here the distribution is general; frequently certain carotenoids are characteristic of one or a group of species. Certainly the carotenoid concentration in green plants varies with the tissue and with age and stage of maturation.

Among the cryptogams, we find wide occurence again, but there are many algae and fungi which produce no carotenoids at all (Mrak, Phaff & Mackinney 1949).

Goodwin (1952) in an extensive review reports that among fungi, beta-carotene is most common, and that lycopene is frequent. Xanthophylls are infrequent, and those which occur are usually acidic.

Work on the formation of carotenoids has been more fruitful; and their biosynthesis has been clearly shown in the long series of experiments by Goodwin and his colleagues, principally on Phycomyces blakesleeanus. This has pointed out the relationships between many of the compounds, being intermediates in several alternative and not mutually exclusive biosynthetic pathways. The remainder may be explained as metabolic derivatives, and the accumulation of one or a few particular carotenoids can be ascribed to a genetical or an environmental enzyme block or switch in the synthetic pathway.

The functions of carotenoids remain a subject of conjecture, and it is partially this which is investigated in the present thesis. In higher plants, as a guide there is the constant association with chlorophyll; and the participation of carotenoids in photosynthesis both in green plants and in photosynthetic bacteria has been demonstrated (Goodwin 1960, Thomas 1950, Clayton 1953). Other suggested functions are phosphorylation (Jagendorf et al 1958) and protection against photosensitivity (Griffiths et. al. 1955), (Sistrom et. al. 1956). These are

discussed at length by Goodwin (1961).

Still, at present the appearance of carotenoids in non-photosynthetic organisms remains apparently fortuitous. Mackinney and Chichester (1960) suggest the role may be a detoxification mechanism, referring to the treatment of the terminal iso-C_3 fragment of leucine. Goodwin and Lijinsky (1951), using Phycomyces blakesleeanus, find a greatly increased carotene accumulation with L-leucine as the carbon source in the absence of glucose.

Because of its' similar absorbing wavelength, carotene has been compared with riboflavin as the receptor in phototropic reactions (Castle 1935); likewise, by comparison of action spectra, it has been suggested as a receptor in photo-oxidations (Thomas 1955). It may be generally stated that a correlation between action spectra suggests a functional capacity but by no means unequivocally proves it. For example, Halldal (1958) working with algae finds both carotenoid-containing and carotenoid-free eye spots are able to mediate phototaxis at the same and at different maxima.

Noting that all the preceding funtions involve some association with light, we are left with the problem that carotenoids occur in relatively large amounts in organisms rarely exposed to light.(Goodwin 1950).

Turning to animals, Goodwin points out that there is a selective accumulation of carotene in gonads, particularly among marine invertebrates.(Fox & Pantin 1941, Goodwin & Taha 1950, Lederer 1935). He suggests here a justification to consider carotenoids intimately concerned with reproduction.

Supporting this view we find that with few exceptions (Kuhn & Winterstein 1950, Karrer & Jucker 1948), carotenoid distribution in green leaves varies little between species; whereas in reproductive structures there are wide differences both in the nature and in the quantities of carotenoids found (von Euler et.al. 1944, Miller & Schomer 1947). These also change with the stage of maturation (Zhukovskii & Medvedev 1949, Miller & Schomer 1947).

Among the fungi, as early as 1918 Lendner reported that there was more pigment in the + strain of Mucor hiemalis than in the - strain (cit. Satina & Blakeslee 1926). Other reports of differential carotenoid accumulation followed. Of particular interest is that the water mould Allomyces produces asexual sporangia devoid of carotene; the sexual plant produces bright orange male gametangia rich in gamma-carotene, and colourless female gametangia (Emerson & Fox 1940). It was suggested (Schopfer 1943) that

+ and - strains of Phycomyces blakesleeanus should have different amounts of carotene as in Mucor hiemalis. This was tested and found to be so (Goodwin et.al. 1951).

Carlile (1956) can find no direct relationship between pigmentation and spore production in Fusarium oxysporum; nor, with Friend (1956), in Pyronema confluens. They suggest that pigmentation and sporulation are unrelated light effects. Burnett (1956) reviewing carotene and sexuality in the Mucoraceae, cannot find direct correlation; too little is known for general concrete conclusions to be drawn.

There is a slight difference in carotene accumulation between + and - strains of Choanephora cucurbitarum, but mixed + and - cultures produce 15 to 20 times as much beta-carotene as do cultures of either strain grown alone (Barnett, Lilly & Krause 1956). This is also true when the + and - mycelia are separated by a cellophane membrane; the effect is reciprocal and is stimulated by diffusible substances. Other members of the Choanephoraceae behave similarly in intra and interspecific crosses (Hesseltine & Anderson 1957). At about the mid-point of this work, a publication was read in which Reichel and Wallis (1958) fail to find a stimulation in mixed + and - cultures of Phycomyces blakesleeanus.

We have, then, many reports of a wide and seemingly fortuitous distribution of carotenoids. Their biosynthesis has been elucidated, at least in fungi. They appear to play some part in photosynthesis, but their function or functions in non-photosynthetic plants and in animals remains in doubt.

Their chemical properties suggest a participation in photo-sensitive reactions, and some evidence has been advanced to support this view. Vagaries of their appearance in fungi upon different treatments of medium and environment suggest a role in detoxification. There is also, finally, their association with sexuality: is it or is it not simply circumstantial?

The present work cannot approach a complete solution to any of these questions, but it does add to the data available concerning each of them; and in doing so it may aid the final elucidation.

Chapter II

EXPERIMENTAL & ANALYTICAL METHODS & RESULTS

EXPERIMENTAL & ANALYTICAL METHODS & RESULTS

A. Fungi Employed

Initial investigations were carried out on Mucor hiemalis strains + and -, available as stock cultures in the Department of Botany of the University of St. Andrews. These were later supplemented by stocks from the Department of Botany of the University of Liverpool, of Phycomyces blakesleeanus strains +1, -1, -6, -E45 and a strain labelled Phycomyces blakesleeanus +H102 which was later found to be P. nitens (see under Experiments). Also obtained were Cunninghamella elegans strains + and - and Absidia glauca strains + and -; all previously from the Department of Botany of the University of Oxford. Originally all the above fungi were obtained from the Centraalbureau für Schimmelculture, Baarn, The Netherlands. Records are unavailable of the date of first subculturing.

B. Cultural Techniques

1. Sterilization of Media and Apparatus

Liquid solutions and often glassware were sterilized by steam pressure of ten pound per square inch for ten minutes. Where dry sterilization was required, articles were heated to 120° C. for at least one half hour; found

to be the minimum effective time.

Prior to inoculation of culture flasks, windows and doors were closed, the air was sprayed with a solution of phenol in methanol, and benches were wiped down with methanol. Care was taken between sterilizing and use and during use to maintain sterile apparatus and instruments, sterile.

2. Stock Cultures and Preparation of Inocula

Fungi were maintained in stock culture on test tube slopes of solid medium consisting of 2% extract of malt (Boots Chemists) and 2% agar. Later, this was replaced with a modified Langeron's medium consisting of 10 gm. old potato and 10 gm. fresh carrot, blended and made up to 1 litre with distilled water (cit. Dade 1960); and as usual solidified with 2% agar. On this medium mycelial growth is sparse but sporulation profuse, and it has the advantage of maintaining healthy cultures for a long time.

For all routine experiments described here an inoculum of spores was employed. This was always in the form of 1 ml. of a suspension in sterile distilled water. A density of about 10^7 was found to yield the highest dry weight of mycelium in Mucor hiemalis; the exact number was not critical within a factor of about 10 either way.

Preparation of the spore suspension was as follows. The fungus was inoculated onto a solid medium, initially malt agar but later potato-carrot agar, in a 250 or 500 ml. conical flask. This was done on a regular basis whenever stocks appeared to be dwindling. The flasks were incubated at 25° C. under illumination.

When an inoculum was required, a culture which was sporulating profusely was selected without special reference to age and shaken with a small amount of sterile distilled water. A few drops of detergent (Teepol) were added to remove the superficial lipids on the spores, making for easier "wetting" and hence quicker germination. After several extractions in this manner, the resulting spore suspension was centrifuged, the detergent solution decanted, the spores rinsed twice in sterile distilled water and taken up in a known volume of the same.

The initial density of the suspension was estimated by means of a haemocytometer, and by appropriate dilution, the inoculum was prepared with the desired density of spores.

Towards the end of this series of investigations a few trials were made using a pre-germinated spore suspension. This was prepared by incubating a dense suspension of spores in the medium to be employed in the subsequent experiment. After 24 to 36 hours, depending on the species, the spores had germinated and

were rapidly metabolising. Such an inoculum eliminated a lag of germination time in experiments by immediately functioning as an active enzyme system. For reasons of comparability with earlier experiments, this type of inoculum was not employed in this series.

3. Experimental Cultures

a. types of media

In all the experiments except those performed on Petri plates, the fungus was grown in submerged shaken culture in a liquid medium. It is this technique which provides the greatest homogeneity throughout the culture, and therefore the best quantitative results and greatest reproducibility (Khuyver & Perquin 1933). The liquid medium also makes for ease in handling and analysis subsequent to culturing.

The medium used was always a simple modification of Schopfer's medium (1934) as follows:

D(+) glucose	5%
DL asparagine	0.2%
$MgSO_4 \cdot 7H_2O$	0.05%
KH_2PO_4	0.15%
Thiamine HCl	0.025 mg.%
Distilled water to	100%

Phycomyces blakesleeanus was the only fungus used which required thiamine (Burnett 1952). Because it was

found to have no adverse effects on the rest, for the sake of uniformity it was always included. The glucose concentration of the medium was widely varied in experiments to determine the optimum carbon to nitrogen ratio.

For all later experiments, 25 ml. of medium per culture flask was employed; dispensed by automatic pipette or by burette.

Where necessary, the medium was solidified with 2% agar sterilized with the medium.

b. methods of aeration.

Fungi grown in submerged liquid culture, having no direct contact with the air, were found to be limited in their rates of metabolism and growth by the rates of oxygen diffusion through the culture medium. This rate of diffusion is slow in standing cultures. Moreover, unless some agitation is provided, the mycelium tends to float and form a surface pad with its' attendant non-homogeneous structure. For these reasons a shaking apparatus is essential to the technique of submerged culture.

Initial experiments were performed utilizing a device, available in the Department of Botany of the University of St. Andrews, which functioned by tilting

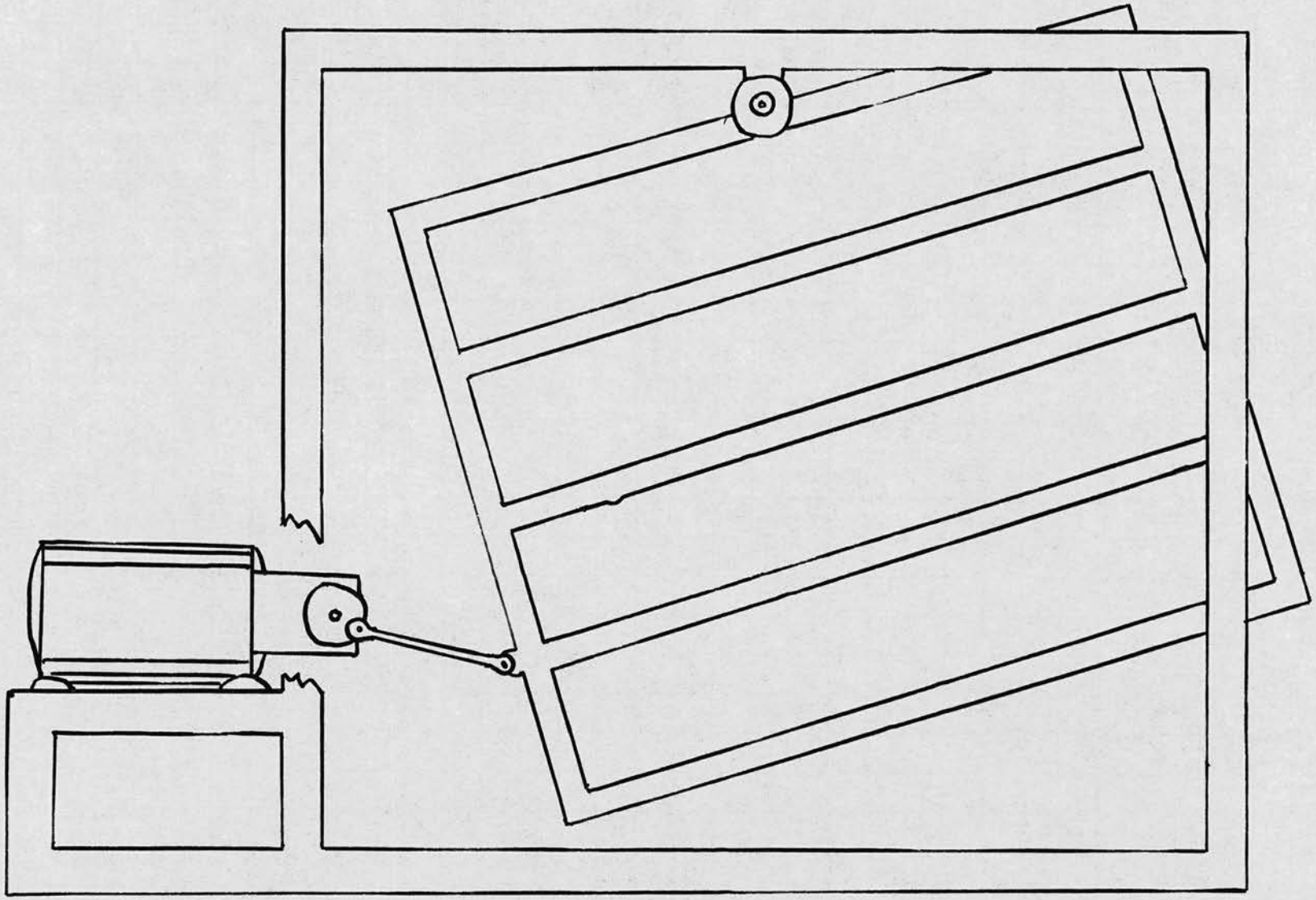


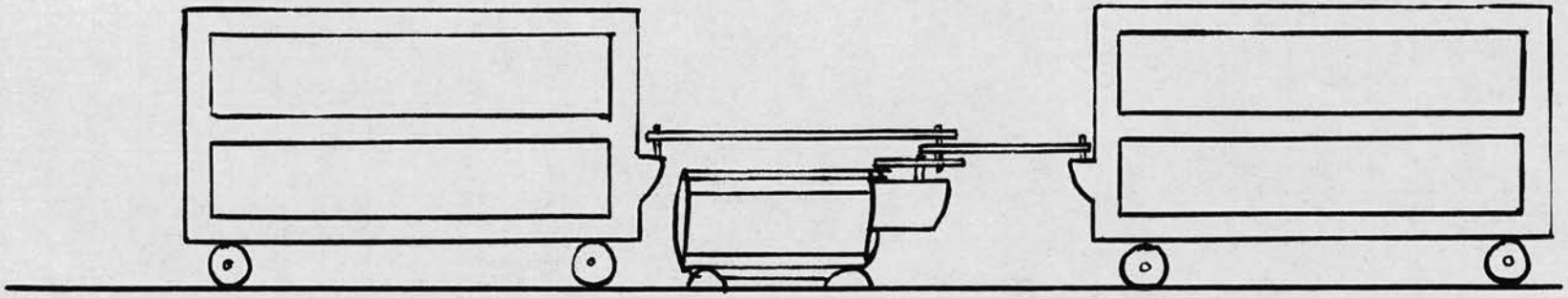
Figure 1 Tilting shaking machine.

the culture flasks (See Fig.1). Now, aeration being a limiting factor to growth, it is imperative that the agitation and hence aeration received by all culture flasks is equal. It can easily be seen that the flasks on the periphery of a tilting device will receive greater agitation than those on an inner radius; it was found that a gradient of dry weight production resulted. For the sake of uniformity of replicate samples and comparability of results, a reciprocating shaker which operated on a horizontal plane was designed and constructed.

Powered by an old wind-screen wiper motor from a B-17 bomber with variable speed control by means of a rheostat, it consisted of two carriages mounted on ball-bearing wheels. Drive was achieved through two connecting rods mounted onto an eccentric by bolting the bearings in a slot, allowing for a variable throw.

The optimum combination for Mucor hiemalis, which also proved satisfactory for the other fungi tested, was found to be a throw of five cm. at about sixty cycles per minute. The majority of experiments were performed using this shaking device (See fig.2).

A similar shaking machine was constructed at Newcastle when a single large carriage was built and



Early version

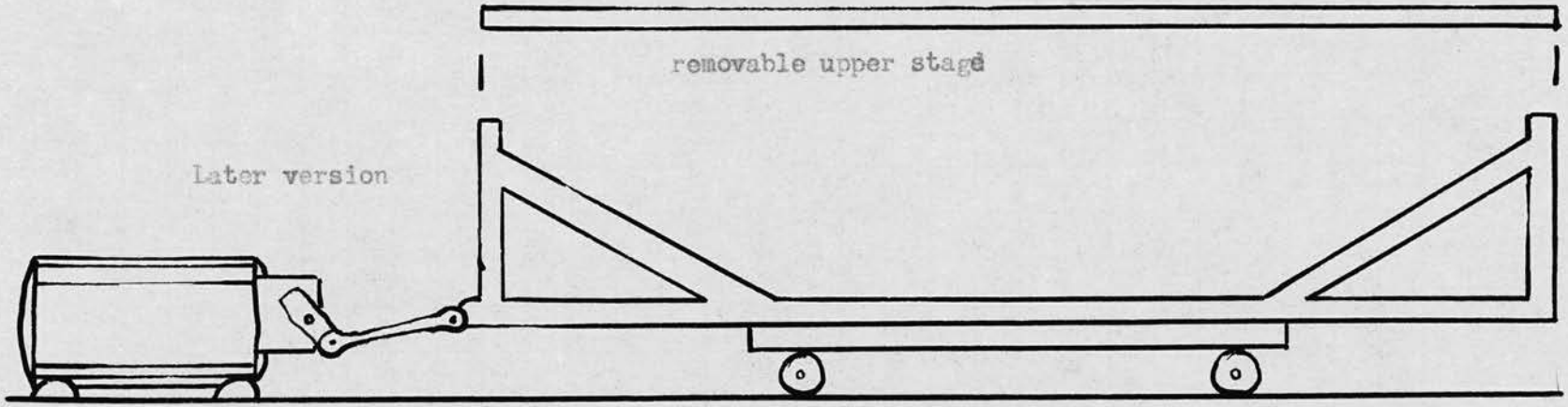


Figure 2 Early and later versions of a horizontal reciprocating shaking machine.

driven by a brushless induction motor fixed at 58 cycles per minute through a reduction gearbox.

Most experiments depended on a sufficient rate of air diffusion through the cotton-wool plugs. However, for experiments involving reduced oxygen tensions, it was necessary to exclude air and introduce a controlled mixture of gases. Attempts were made to employ sintered glass, gas distribution tubes inserted through two-hole rubber stoppers. These were found satisfactory up to three to five days growth, when the strong aerotropism of the fungi blocked the tubes with mycelium so thoroughly as to make them useless.

Since three to five days is not a long enough period for adequate physiological ageing, an alternative method was sought. Open-ended glass tubes were tried but the same difficulty was encountered, together with the necessity of arranging all the tubes at the same depth below the medium to ensure uniform distribution rates. A new technique was still desirable.

Since gases are soluble in liquids in proportion to their partial pressures above the liquids; if culture flasks are shaken to establish a high rate of diffusion, in order to maintain a controlled

oxygen tension in solution, it is necessary only to provide a constant mixture above the medium. This was accomplished by passing a continuous flow of the desired mixture of oxygen and nitrogen through the culture flasks. Flasks may be arranged in parallel or in series. Obviously parallel is best because it eliminates build-up of waste metabolic gases, and small-scale experiments were performed in this way.

In practice, parallel gas distribution becomes cumbersome due to the need for a separate gas delivery tube for each flask. By analyzing the effluent gas from a series of five culture flasks with a paramagnetic oxygen analyzer (Beckman), it was found that a very low flow rate maintained a constant oxygen percentage. Large-scale experiments were set up in this manner (See frontispiece).

Gases were supplied by the British Oxygen Company. Cylinders of 5% oxygen and 3% oxygen in nitrogen were supplied ready-mixed. Lower percentages were derived from nitrogen by introducing minute quantities of air.

c. methods of illumination

Early tests were not provided with special illumination but relied upon existing lighting in the controlled temperature room. It has been reported

(Goodwin 1950) that light has a marked stimulatory effect on carotene accumulation in Phycomyces blakesleeanus, and it was found to have a similar effect on Mucor hiemalis.

Initial experiments using two stages of the tilting shaker showed discrepancies in carotene percentages as well as in dry weight. An experiment designed to investigate the effect of light demonstrated a nice gradient of carotene percentages which decreased with increasing distance from the light source.

In subsequent experiments, lighting was therefore provided over each carriage by two 60W. incandescent lamps with aluminium foil reflectors and muslin (gauze) diffusion screens.

The Newcastle shaking machine was lit by two four-foot fluorescent bulbs along the length of the carriage.

The light sources used generated appreciable heat, so a fan was set up to dispel the heat and the thermostatic control for the constant temperature room was adjusted to result in an operating temperature of 25° C. within the shaking apparatus and lighting system.

Experiments directly investigating the effects on mycelial growth and carotene accumulation of light versus dark required darkened conditions for culture flasks, other conditions being the same. This was

achieved by painting culture flasks black, and then silvering or covering them with white paint, to prevent differential heat absorbtion. Such darkened flasks were incubated on the lower stages of the carriages, which, as a further precaution, were enclosed with black cloth.

Some trials were made employing light of different wavelengthsby enclosing the light source within screens of plastic material whose optical transmission was known. Due to lack of time, these experiments were not extensive and since they were inconclusive they are not reported here.

C. Chemical Analyses

1. Determination of the pH of Media

It can be seen that in the experiments with reduced oxygen tensions, there will occur a critical oxygen level at which metabolism is half aerobic and half anaerobic. Above and below this level there will be differential accumulation of the metabolic products of the two pathways, with an accompanying alteration of pH. Thus the pH of the remaining medium can be a meaningful value, of use in interpretation of other results.

For the determination of pH, a small aliquot of

the residual medium in the culture flask was decanted prior to analyses for any other factor. The pH of this aliquot was measured potentiometrically with a Cambridge (Pye) pH meter. The aliquot was then returned to the culture flask along with a single distilled water rinse.

2. Determination of Dry Weight

Following an appropriate period of growth, the total contents of the culture flask was decanted through Whatman No.4 filter paper under suction in a Büchner funnel. The flask was rinsed twice and the rinsings added. The whole mycelium was then rinsed twice with distilled water to remove traces of the nutrient medium.

The compacted pad of mycelium was dried to a certain extent by the applied suction, then carefully lifted entire from the filter paper and deposited into a tared 50 ml. Pyrex Quickfit conical flask. The choice of vessel was to enable all routine determinations to be performed on the sample with as few transfers as possible, thus keeping experimental error at a minimum.

Upon completion of other determinations, the majority of the solvent, acetone (see later), was evaporated off by means of an electric hot plate. The flask was then dried for three hours or more at 80° C. It was found that further drying did not affect the weight

by more than 0.5 mg. Since variation between duplicate samples was appreciably greater than this, it was deemed unnecessary to dry to constant weight.

Early experiments were analyzed by extracting the mycelium with fat solvents; then drying and weighing the insoluble remainder while on the filter paper. This was found unsatisfactory because of the several transfers involved and the unpredictable loss of weight upon drying of the filter paper. In addition, this technique gave the net dry weight without carotene, lipids, and other acetone-soluble components; and required a further determination of these, introducing more sources of error.

Dry weights given are in milligrams, and express total, overall dry weight. It is upon this basis that the weight-percentages of carotene are calculated.

3. Determination of Total Carotene

a. extraction of the medium

Because carotene is insoluble in water or in aqueous salt solutions, it was considered unnecessary to make extraction of the medium a routine process. However, a disadvantage of the shake culture technique is that older cultures tend to be auto-digested, or to dissolve into the medium, leaving a thin liquid. Thus, in

long-term experiments, droplets of lipoid material rich in carotene appeared, beginning on about the ninth day, floating on the surface of the medium. These presumably arise from such auto-digested mycelium.

For this type of experiment, the medium was extracted by a phase distribution technique between water and light petroleum of the 8 to 10 carbon range, boiling point 40 to 60° C. Three extractions were made of each sample as follows: Shake a small quantity of the solvent (10% v/v) with the medium in a separating funnel. Allow the two phases to separate. Draw off the lower, water phase and run the solvent phase into a volumetric flask of appropriate size. Return the water phase to the separating funnel and repeat the process. A final rinse (solvent) of the separating funnel was added to the combined extracts and the total made up to volume. A separate optical reading was made; later added to that of the mycelial extract.

b. extraction of the mycelium

Following Goodwin (1950), first attempts were on the principle of completely drying the mycelium by some means, taking care to avoid destruction of the carotene, and then extracting with a suitable carotene solvent.

After much experimenting with various drying agents (anhydrous salts, sulfuric acid, alcohols...) and various solvents (diethyl ether, light petroleum, acetic acid...) and combinations of drying and extraction in one process, the following technique was developed and adopted for all routine analysis.

Acetone was selected as the most suitable solvent because of its complete miscibility with the small amount of moisture invariably remaining in the mycelium. It was also found to have very nearly as high a capacity for carotene solution as diethyl ether or light petroleum, the best solvents tried. In any case, no sample encountered contained carotene approaching the limit of carotene solubility in the final resulting mixture of acetone and water.

As mentioned under dry-weighting procedures, the first technique involved repeated extractions of the mycelium upon filter paper. This technique introduced sources of error and was inconvenient in the time required for analysis of each sample.

Upon acquisition of a supply of Quickfit glass-stoppered conical analysis flasks, a simpler, more precise technique evolved. After filtration and rinsing, the pad of mycelium was placed in the tared flask,

weighed, and a volume of acetone added. From experience, a volume of 10 to 20 ml. depending on the sample generally gave a reading of optical density within the range of greatest precision for the instrument employed.

After addition of the solvent, the flask was tightly stoppered and left to stand in the dark at room temperature for a period from one to three hours. This allowed complete diffusion of the carotene content of the mycelium. It was found that vigorous shaking lessened the diffusion time but so disrupted the mycelial pad that it became difficult to obtain a clear aliquot for analysis. Standing in the dark at room temperature for up to eighteen hours caused no measurable depreciation in the optical density of the extract.

The analysis flask plus contents was weighed immediately prior to pipetting an aliquot for analysis. In routine work the aliquot was returned to the analysis flask following inspection, for determination of dry weight. From these figures (flask tare, flask+wet mycelium, flask + mycelium + solvent, flask + dry mycelium), it will be seen that all necessary weights of acetone and water (and dry weight of mycelium) can be obtained. The total volume of solvent can then be calculated, noting the density of acetone at room temperature.

This technique involves but two transfers; namely from filter paper to flask and from flask to spectrophotometer cuvette. With reasonable care, both may be accomplished without prejudice to dry weight or to the weight-percent of carotene.

After some months practical use, a paper by Jensen (1959) was encountered which described a technique for extraction of carotene from seaweed using acetone as the principle solvent. He found higher overall recovery of carotene using this technique than was obtainable by the conventional analysis of Haug & Larsen (1957).

c. spectroscopic examination of the complete extracts

The nature of the carotene molecule and the minute quantities involved make the inherent qualities of optical analysis the best method for its quantitative determination. The principles follow the laws of Lambert, Bougier and Beer, which essentially state that the absorbtion of light by a coloured solution varies with its density and thickness. A more complete discussion of the chemistry of the carotenoids is presented by Goodwin (1952) and need not be included here.

Beta-carotene was definitely recorded in both Phycomyces blakesleeanus and in Mucor hiemalis by Schopfer as early as 1930. It has since been confirmed that it is the principle pigment, by many workers.

Absorbtion curves in acetone of the pigments extracted from present experimental cultures showed peaks at 455 mmu and inflections at 405 to 410 mmu and at 470 to 480 mmu. These correspond to but are displaced by a few mmu from a standard absorbtion curve for beta-carotene in hexane.

The peak in acetone at 455 mmu was corroborated by a report of a peak for beta-carotene in acetone at 456 mmu by Jensen (1959). An extinction coefficient was not calculated. Rather a coefficient of 2550, the mean of several reported extinction coefficients for beta-carotene in hexane, was used. Later, upon encountering Jensen's paper, all results were recalculated on the basis of his report of an extinction coefficient for beta-carotene in acetone of 2490.

For all routine analyses, the optical density of an aliquot of the sample was directly read at 455 mmu. One cm. glass cells were used and readings were against a blank of acetone containing 3% v/v distilled water. The instrument was a Cambridge Instrument Company Unicam SP 600, equipped with current stabilizer and interference filter.

d. expression of the final result

Because of the elaborate and time-consuming work

involved, the mycelial extract was not further purified as a routine procedure; in accordance with common practice (Garton et.al. 1950, Barnett et.al. 1956, Hesseltine & Anderson 1957). Thus in most experiments results are expressed as weight per cent of "total carotene", in micro-gm per gm dry weight of mycelium. It will be seen from the foregoing that "total carotene" is that fraction of the acetone-soluble portion of the mycelium which absorbs light at 455 mmu.

As found by other workers (Hesseltine & Anderson 1957), there was a loss of 5 to 15% of the optical density of a particular sample during saponification. Chromatographic separation of the purified sample showed that less than 5% of the colour was not beta-carotene (see later), as was also found by Garton et.al.(1950). Therefore, "total carotene" is about 10 to 20% more than actual beta-carotene, assuming 100% recovery in the purification procedures. This latter assumption is justifiable since Jensen (1959) reported recovery of beta-carotene of 97 to 100%, using his technique.

4. Breakdown of the Mycelial Extract

a. saponification of the complete extract

This was carried out after the methods of Goodwin & Morton (1946) with modifications as follows.

The acetone extract containing the carotene was reduced in volume to about 10 ml and an equal volume of 60% aqueous KOH added in a separating funnel. The mixture was covered with nitrogen, shaken vigourously and left to stand in the dark for 12 to 18 hours. After this time it had separated into two phases; the lower principally water containing KOH and soaps, the upper principally acetone containing carotene.

The carotenes were extracted from this mixture as described in extraction of the medium; i.e. with three portions of light petroleum. Tests showed a loss of colour during the saponification procedure of 5 to 15%.

b. chromatographic separation of the carotenes

i. column chromatography

The carotene solution is rinsed with distilled water to remove any remaining soaps and is then chromatographed onto a column of alumina after Carton et.al.(1951). A glass column 20 cm by 1.8 cm drawn out to a capillary was used. It was shrouded with black paper to avoid or minimize oxidation of the carotenes during development of the chromatogram.

Mixtures of light petroleum and 1 to 5% acetone were tried for development. The precise ratio appeared to

make little difference to the rate or the sharpness of development. The carotenes resolved into two coloured zones, the lower one much larger than the upper. These turned out to be respectively beta-carotene and alpha-carotene.

Under inspection with ultraviolet light, a fluorescent colourless zone was sometimes observed between the two coloured zones. This has been to date invariably lost in attempts to elute for further examination.

ii. paper chromatography

After encountering Jensen's (1959) paper, and soon afterwards Jensen & Liaaen Jensen (1959), no further work was done with columns as this was generally cumbersome and time-consuming. In addition, paper chromatography lessens the need for saponification since the lipids are carried with the solvent front.

The technique, following Rutter (1948), is as follows. The chromatogram is circular on a kieselguhr-filled filter paper (Schleicher & Schull, no.287, kieselguhrfilter, 18 cm diameter), kieselguhr content about 20%. Two parallel slits about 2 cm long are cut from the centre along a radius; the width, which depends on the desired rate of chromatogram development, is from 1 to 5 mm. The outer end is cut and the strip so formed

is folded down to act as a wick for the developing solvent, to rise out of a Petri plate.

An aliquot containing about 20 micro-gm of carotene is applied with a capillary to the centre, at no time allowing the "damp" spot to grow larger than a few millimetres in diameter. Jensen recommends applying a small amount of acetone to the wick before development as an aid to producing more regular zones.

The spot of carotenes is developed as in column chromatography with a light petroleum and acetone mixture. The circular paper is placed over a Petri plate containing the developing solution with the wick projecting into it. Good separation of the two coloured zones took 15 to 30 minutes in a covered dish. The procedure is best carried out in the dark to minimize oxidation. With simple carotene mixtures, it is not necessary to calculate Rf values, and close temperature control is not essential.

Upon good separation, and without allowing the chromatogram to dry, the zones are rapidly cut out with scissors and the paper is packed into small columns. Fractions are eluted with acetone and made up to known volumes to be spectroscopically examined.

This technique was tested with and without pre-

saponification of the extracts. The lipids migrated at approximately the same rate as did the beta-carotene and interfered little with the regularity and separation of the zones. The resulting eluants varied slightly, giving optical densities of from 1 to 3% higher when lipids were present in the beta-carotene fraction than when they were saponified and removed, after the chromatographic separation. The small difference suggests, when compared with the 5 to 15% loss on prior saponification, that some acetone-soluble component which absorbs at 455 mmu remains adsorbed on the kieselguhr paper.

c. spectroscopic examination and identification of the eluted fractions

Each carotene fraction was characterized by its absorbtion spectrum. Standard curves are those of the compound dissolved in hexane rather than acetone. Acetone curves were of the same shape but were always displaced by a few mmu towards shorter wavelengths. Most tests were made in acetone, but as a check, samples in acetone were evaporated (under nitrogen to minimize alteration) and taken up in hexane for examination. The resulting curves were very close to accepted curves for the compounds. Discrepancies were probably due to small amounts

of impurities.

Readings of optical density were taken at intervals of 5 mmu over the range from 380 mmu to 520 mmu. In critical regions i.e. surrounding peaks and points of inflection, readings were taken at intervals of ½ mmu in order to position these correctly. In each case, the critical points were rather less sharply defined, but corresponded to the recognized standard absorption spectrum for a particular carotenoid.

Since such spectroscopic inspection is now generally accepted as a precise qualitative analytical technique, and since results agreed with those of other workers to a large extent, these absorption spectra were considered sufficient evidence for identification of the compounds involved. Attempts were made to crystallize some of the by slow evaporation of a sample in ethanol, but these have so far been unsuccessful.

d. expression of the final result

In experiments where a complete analysis of the extracts was made, total carotene is given as usual in units of micro-gm per gm dry weight of mycelium. Each fraction is given both in the same units and as a percentage of the total carotene. Where the figures do not add up to 100% there are two probable causes: a

slight loss during the purification technique and some absorbtion by an undetected colourless substance in the determination of total carotene.

D. Experiments and Results

As can be seen from the preceeding chapter, methods of culture and analysis were more or less continuously being developed. With each major alteration in technique, the possibility arose of altered results, and all the previous experiments were repeated using the new technique. Generally, results turned out relatively the same but with an improvement in the precision of replicate samples. Following the establishment of a satisfactory general technique, each experiment was repeated at least once on the standard basis. Where several experiments employed the same conditions, they have sometimes been lumped together as one.

For ease in reading, comparison, and reference, extracts of the results for each experiment are expressed in general terms. Relative figures are given as percentages. Complete detailed tables of data and analysis are given in the Appendix.

The experiments fall into four groups, which are presented in their order of logical consequence. The

initial impetus to the work was to investigate other members of the Mucoraceae for a stimulation of carotene production by mixed plus and minus cultures; as was described by Barnett et al (1956) in Choanephora cucurbitarum. To do this, the techniques earlier described had to be developed; and this comprised a considerable portion of the year's work.

Initially it was of interest to compare the carotene production of various species and strains of the Mucoraceae. No great differences, and no significant stimulations in mixed cultures were found. Attention was therefore diverted to other aspects of carotene production. In particular, the work of Cantino et al (1953, 1956, & 1957) on Blastocladiella directed interest towards conditions of illumination and of changes in the gas phase.

The intention of the first group of experiments, into methods and techniques, was to discover the optimum medium for each fungus studied, and from there to solve each technical problem as it arose.

1. Experiments into Methods and Techniques

EXPERIMENT 1- Experimental culture medium

a. The criterion chosen by which to judge the merits of a particular medium was the growth rate of the fungus on said medium. In this experiment, the fungi tested were Mucor hiemalis strains + and -, Cunninghamella elegans strains + and -, and Absidia glauca - strain. Schopfer's glucose-asparagine medium was made up as usual except that the glucose was added separately to make up percentages of 1,2, 5, 10, and 15%. The medium was solidified with 2% agar and dispensed on 10 cm diameter Petri plates. Each medium was prepared in duplicate for each fungus, making a total of fifty plates.

The plates were inoculated by transferring a portion of a rapidly-growing colony of the fungus. They were cultured in darkness at 25° C. Growth rate was measured simply by taking the colony diameter at noted time intervals. (See Table 1).

All the fungi tested showed most rapid increase in colony diameter up to 97 hours on media containing 5 to 10% glucose, with the exceptions of Mucor hiemalis + strain, which showed fastest increase at 10 to 15% glucose, and Absidia glauca - strain, which showed fastest

increase at 2 to 5% glucose.

The type of growth on the different glucose concentrations varied. In particular, both strains of Mucor hiemalis showed much more luxuriant growth on media containing 2 to 5% glucose than on those of higher glucose content, though these colonies were increasing in diameter more rapidly. In addition, the faster growing specimens completely filled the Petri plates with mycelium in as little as 2½ days. This length of time is not sufficient for experimental cultures to accumulate an apreciable amount of carotene.

For these reasons a further test was designed wherein growth was measured by dry weight of mycelium.

b. Experimental submerged shake culture technique was used to determine growth rates on media containing several different carbon concentrations. Media were prepared containing glucose concentrations of 0.3, 1.0, 3, and 10%. The fungi tested were Mucor hiemalis strains + and - and Phycomyces blakesleeanus strains +1 and -1. They were cultured in 25 ml portions of liquid medium in 150 ml conical flasks in submerged culture, aerated by shaking. Flasks were inoculated with a suspension of spores. Growth was for 7 days, the normal experimental period.

(See Table 2)
Duplicate results showed that both greatest dry weight and greatest weight percent of carotene was produced by colonies grown on the media containing 10% glucose. There was an apparently linear decrease through the other media to dry weight and weight percent of carotene produced on media containing 0.3% glucose of approximately one half of the values produced on media containing 10% glucose.

Media containing 3% glucose supported growth and carotene accumulation only about 8% less than media containing 10% glucose. Higher percentages in general of glucose (and other organic constituents) in media raise complications during sterilization by autoclaving (Hall 1959). For these reasons, a medium containing 5% glucose was adopted for routine experiments. (See Table 1)

EXPERIMENT 2- Sterilization techniques

a. As recommended by Schopfer (1934) and followed by Garton et.al. (1950), the acid salt in the medium, KH_2PO_4 was, to prevent precipitation of the phosphate, sterilized separately from the other components and mixed upon cooling. This necessitated sterile dispensing of the complete medium into sterile culture flasks, as opposed to first dispensing and then sterilizing.

Because of the inconvenience and the introduction

of uncontrollable amounts of water condensate during the autoclaving, dry oven sterilization of glassware was tried.

Culture flasks were washed and rinsed as usual and plugged with gauze-covered cotton-wool. Six flasks were heated to 120° C. for each of ½, 1, 2, 4, and 18 hours; a total of 30 flasks. After cooling, 25 ml of 5% glucose-asparagine medium was dispensed by sterile automatic pipette into each flask, beginning with those heated for the longest time. The flasks were then incubated at 25° C. on the shaking machine, under illumination.

There was no growth visible unaided after 1 or after 2 days incubation. After 4½ days, all flasks were carefully inspected. (See Table 3). There were seven flasks uncontaminated, all of which had been heated for 2 hours or less and all were among the last 13 to receive medium. Twenty flasks of which half had been heated 4 hours or more and were the first to receive medium, contained a single fungal contaminant. Two flasks each contained two fungal contaminants; and one, many fungal and also bacterial contaminants. These had been heated 18 hours but were the first to receive the medium.

It was clear that the most serious source of contamination was during the attempted sterile dispensing of medium, necessitated by separate sterilization of the acid

phosphate. The best method of eliminating the contaminants is to eliminate the source. This can be done by sterilizing the medium in toto, and a test was run to determine if it is really essential to sterilize the phosphate salt separately.

b. From reference to Hall (1959) an interaction between the amino acid component and the glucose was suspected as a result of the conditions under autoclaving. To determine whether this occurred to a large extent with the medium in use here, a range of times of autoclaving was tried. The total medium was autoclaved at 10 lb pressure for times of 5, 10, 15, and 30 minutes. Samples were in replicates of six. A control was provided of samples prepared by separate sterilization and subsequent dispensing. The testing criterion was dry weight of mycelium. Half the samples were inoculated with a spore suspension of Mucor hiemalis - strain, and half were not inoculated as controls for the degree of sterilization.

The only samples not found completely sterile were two of those autoclaved for 5 minutes: these contained some unidentified bacteria which may have been later introduced. However, those autoclaved for only 5 minutes which were sterile supported the best growth of the fun-

gus. The other samples produced a decreasing dry weight with increasing time of autoclaving, to a minimum at 30 minutes of 11% less than that at 5 minutes. (See Table 4).

There was no significant difference between the medium sterilized in toto and that sterilized separately.

EXPERIMENT 3- Inoculum size versus dry weight

a. In the early stages of the work, it was suspected that some of the anomalies in results might be due to differences in the numbers of spores in the inocula. This was put to the test by the simple means of diluting a spore suspension of known density into a decreasing series of inocula, and dry weighting the resulting cultures after the standard growth period of seven days.

Densities were prepared at 10^8 spores per ml and ½, ¼, 1/10, and 1/100 of this by dilution. Mucor hiemalis + strain was used. Culture conditions were liquid 5% glucose-asparagine medium, incubated at 25° C. under illumination on the shaking machine. Samples were prepared in triplicate.

The greatest dry weight was produced by the inoculum of 10^7 spores. The greater and the lesser inocula produced dry weights of 20% less.

b. A similar experiment was performed with the - strain. Results were proportionately similar, with the greatest dry weight produced by an inoculum of 10^7 spores (See Table 5). It was made clear that the + strain was considerably faster-growing than the - strain. The dry weight produced was of the order of 80% greater than that produced by the - strain under identical conditions.

EXPERIMENT 4- Shape of culture flasks

a. To increase the capacity of apparatus, more 100 ml conical culture flasks were obtained; at an intermediate stage of the work. These were of a slightly different shape from the culture flasks already in use and were tested to discover whether or not they had a different effect on growth.

Mucor hiemalis - strain was used for the test. Conditions of culturing, medium, and growth were standard. Since any difference was likely to be small, six replicates were prepared.

It was found that dry weight was reduced from that produced in the old flasks by about 30% (See Table 6). Aeration was probably the limiting factor since the new flasks were taller and narrower, and the surface of the medium was correspondingly less. Total carotene accumulation/

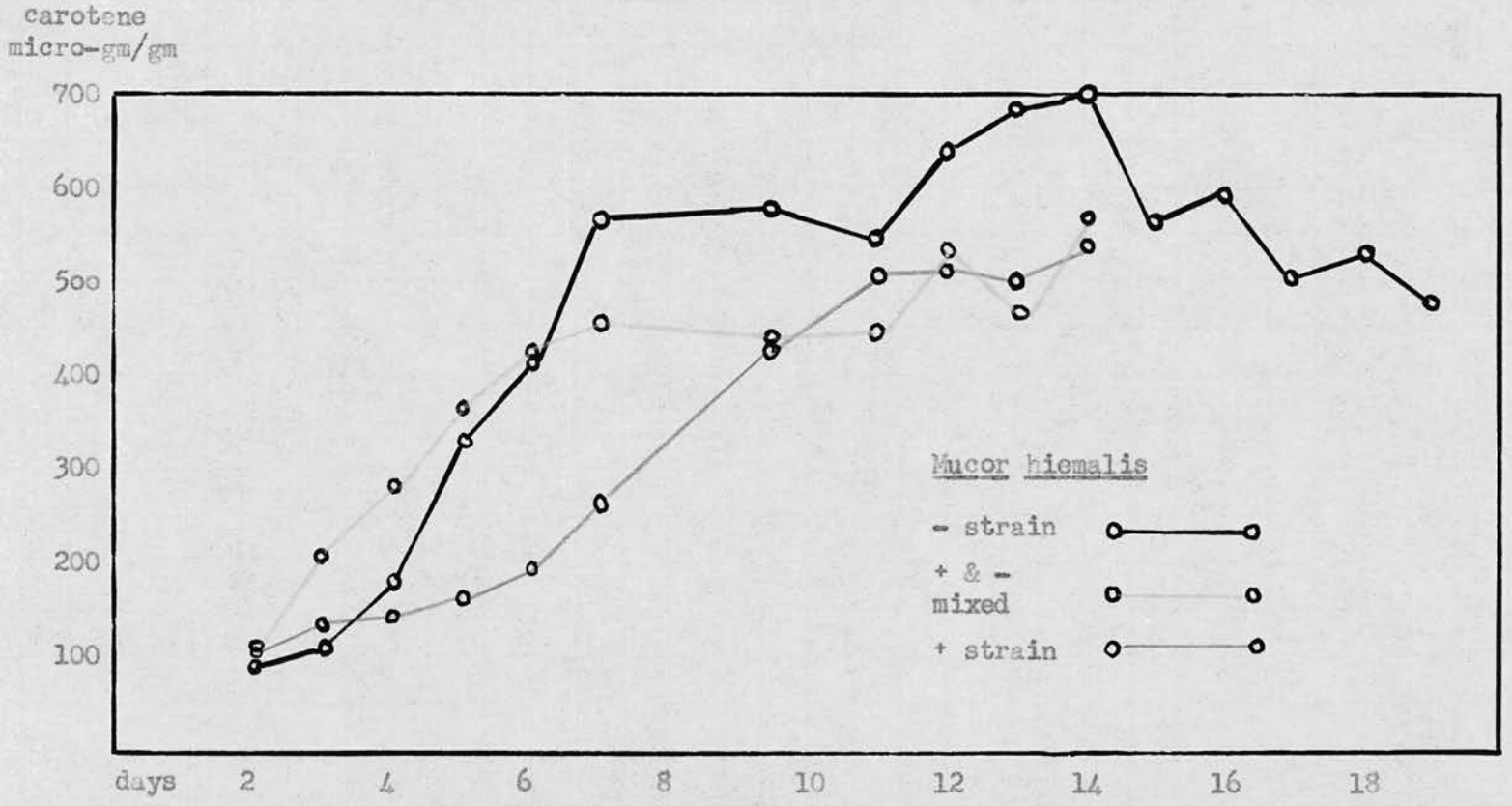


Figure 3 Carotene accumulation versus time.

lation was also less. However, when calculated to the basis of micro-gm per gm dry weight, relative accumulation was the same.

b. Upon transferring work to the laboratories of King's College, Newcastle upon Tyne; where the culture flasks in common use are 150 ml, a similar test was performed.

Results were comparable (See Table 6). Both the dry weight and the carotene accumulation increased by 25%; resulting in a calculated carotene in micro-gm per gm dry weight not significantly different. The test was not performed with every strain of fungus used.

EXPERIMENT 5- Strain tests of Phycomyces blakesleeanus

a. Mating tests. Five strains labelled Phycomyces blakesleeanus were received, numbered as +1, -1, +H102, -E45, and -6. To check the virility i.e. the capability to produce zygospores, of each strain, theywere mated in all possible + versus - crosses.

Spores were streaked about two cm. apart on solid 2% malt extract agar in 5 cm Petri plates. Tests were made in triplicate. The rates of growth of all strains were essentially equal. After two days, the hyphae of both inocula were intermingling and some asexual sporangiophores were in evidence.

With the exception of strain +H102, knots of hyphae appeared along the junction between the two strains on the third day. By the fourth day, tong-shaped suspensors had arisen above the medium; and by the end of a week, all the crosses (except those involving strain +H102) had produced many black, heavily adorned zygospores.

b. The anomolous strain +H102 was immediately suspected of being a different species. Accordingly, spores of each strain were measured, length and breadth.

Taking the means of twenty measurements of each dimension, the spores of strain +H102 were 130% longer, and 60% wider than the means of corresponding dimensions of all five other strains (See Table 7).

Naumov (1939) quotes spore sizes as follows:

For P.blakesleeanus Burgeff 1925: 10-18 by 8.3 mu
For P.nitens (Agardh 1817) Kunze 1823: 18-30 by 8-15 mu

Actual measurements on current cultures are:

Strain +H102: 18.3-34.3 by 9.2-14.2 mu
All other strains: 10-15.7 by 6.7-10 mu

These figures correspond nearly exactly with Naumov's quoted figures; indicating that strain +H102 is in fact Phycomyces nitens and the other strains, P.blakesleeanus.

EXPERIMENT 6- Time versus carotene accumulation

a. For purposes of comparison only, it may be sufficient to simply run experiments for the same lengths of time. There is the possibility, however, that alteration of an environmental condition has no direct effect on the process under consideration (in this case, carotene accumulation) but merely changes the rate of physiological ageing. For this reason it was necessary to establish a normal curve for time versus carotene accumulation.

Mucor hiemalis +, -, and mixed + & - cultures were tested. Culture conditions were standard: 25 ml of 5% glucose-asparagine medium in 100 ml conical flasks plugged with gauze-covered cotton-wool. Growth was in submerged shake culture at 25°C., illuminated. Samples were in duplicate or triplicate.

Because of the limitations of the apparatus, the experiment had to be performed in parts. That is, one set of samples was grown and sampled over the first week; then another set of samples was grown for a week before sampling was started, and so on until a peak carotene-to-dry-weight ratio was found. Sampling for the first week was at one-half day intervals and thenceforth at daily intervals. The experiment was carried for 19 days.

The peak level of carotene to dry weight, about

seven hundred micro-gm per gm dry weight under these conditions, occurred at 14 days (See Figure 3, Table 8); after which it gradually dropped off.

This experiment indicated the relative rates of accumulation of carotene by the two strains and by mixed cultures. It appears that mixed culture increases most rapidly, but also levels off first.

b. Dry weight (See Table 9) increases rapidly to a near-maximum after two days, when it remains fairly constant.

2. Experiments into Species and Strain Differences

The previous group of experiments established optimum cultural conditions and periods of growth. Before it could be discovered if a stimulation occurred in mixed cultures, the carotene accumulation of isolated strains had to be established. Once this was done, the carotene accumulation of various intra and inter-specific crosses was tested.

EXPERIMENT 7- Species and Strain differences

Following the reports by Barnett et al (1956) and by Hesseltine & Anderson (1957) of a stimulation of carotene accumulation in mixed + and - cultures of some of the Choanephoraceae; it became desirable to discover whether this is a general or an isolated phenomenon/

menon . The experiment here described was in fact several experiments covering the various species and strains illustrated. The procedure used was identical in every case so they are included together.

For standard results, growth was on 25 ml of 5% glucose-asparagine medium in a 100 or 150 ml conical culture flask plugged with gauze-covered cotton-wool. The inoculum was a 1 ml portion of a spore suspension of 10^7 spores per ml, found to be optimum. Cultures were incubated at 25° C. in submerged, liquid shake culture for seven days; and analyzed in the usual way described earlier. Samples were usually in triplicate.

Among the singly-cultured strains, the following accumulated carotene to relative amounts (micro-gm per gm dry weight of mycelium) in the order listed: (See Table 10).

Mucor hiemalis -
Mucor hiemalis +
Phycomyces blakesleeanus +1
P.nitens +H102, P.blakesleeanus -1 and -E45; equal
P.blakesleeanus -6

None of the strains of Cunninghamella elegans or of Absidia glauca accumulated carotene to an appreciable degree. Mycelial extracts generally showed an optical density of the order of 0.03 at 455 mmu, which if due

to carotene, would be about 2 micro-gm per gm dry weight on the basis of the dry weight of the mycelium extracted.(See Table 11).

However, there was no apparent yellow colour to the extracts; which strongly resembled in appearance and in optical properties, extracts of very young cultures of Phycomyces blakesleeanus and Mucor hiemalis, probably before they had begun to accumulate carotene.

The absorbtion spectra of none of these extracts resembled that of any familiar carotenoid; certainly not that of beta-carotene. The slight turbidity was probably due to lipids or some partially soluble suspended material. Possible some colourless polyene was present.

The tests of Cunninghamella elegans and of Absidia glauca were extended over a growth period of 28 days. Dry weight of mycelium increased gradually and continuously over this period, but the optical density of the mycelial extracts remained the same at the end of the test, from the oldest culture, as after three days growth.

b. All possible intra- and inter-specific strain mixed cultures were tried for possible increased carotene accumulation. The procedure was as in the preceding tests. Inoculum was one half + spores and one half - spores.

In all such crosses, the final calculated carotene

accumulation in micro-gm/gm dry weight of mycelium was always intermediate between the values accumulated by the component strains grown singly (See Table 12). It is interesting to note that in very few cases is there an increase over the theoretical value, the arithmetic mean of the accumulations of the component strains; and in as many crosses there is a decrease.

These results are in agreement with the findings of Reichel and Wallis (1958). In tests of mixed cultures of their strains of Phycomyces blakesleeanus +1 and -1, +3 and -3, and +4 and -4; they found no stimulation of carotene production over cultures grown singly.

Thus it is clear that the enormous increase in carotene production found in mixed cultures of Choanephora cucurbitarum by Barnett, Lilly and Krause (1956) does not occur in the fungi examined here. It remains to be looked for more widely, but such a stimulation at present appears to be confined to the Choanephoraceae.

3. Experiments into Effects of Varying Illuminations

Following the negative results of the preceeding group of experiments, interest turned to environmental effects on carotene production. During some earlier trials it had been noticed that cultures farthest from the light source were clearly less pigmented than those nearest. This proved to be a well-defined phenomenon, as the following experiments show.

EXPERIMENT 8- Gradient of light effect.

Many workers have reported a stimulatory effect of light upon carotene accumulation by several different species of fungus (Haxo 1949, Goodwin 1952). No direct reports of a light effect upon Mucor hiemalis have been found. Goodwin et al (1950, 1951) state that Phycomyces blakesleeanus cultured in the dark produces about one half the amount of carotene produced under similar conditions in the light.

An experiment was designed to discover the degree of light effect upon Mucor hiemalis. A 500W reflecting incandescent lamp at a height of 18 inches was used as a point source of illumination. The - strain of the fungus was cultured in 500 ml conical flasks containing 100 ml of liquid 5% glucose-asparagine medium; aerated by bubbling/

bling cleaned, sterile air through sintered glass, gas distribution tubes inserted through two-hole rubber stoppers. Incubation was at 25° C. for five days.

The flasks were arranged at distances of ½, 1, 2, 4, and 8 feet from the base of the lamp support, on alternating sides. By this means, each flask received approximately one quarter of the illumination received by the preceding flask. Samples were in duplicate.

Upon analysis in the usual manner (described earlier), results were quite regular. (See Table 13). There was little divergence from the following pattern: Each culture produced about 90% as much dry weight of mycelium and 80% as much carotene, micro-gm per gm dry weight, as the preceding culture. The culture nearest to the light source is taken as 100% in both cases.

EXPERIMENT 9- Dark versus light effect

a. This experiment was repeated a number of times under different conditions of aeration; including bubbling through sintered glass, gas distribution tubes, and through open-ended glass tubes; and shake culture.

In all cases the medium was liquid 5% glucose-asparagine (modified Schopfer's 1934) in different quantities. For each test, "light" and "dark" cultures were grown

side by side in painted and unpainted flasks. Mucor hiemalis + and - strains and Phycomyces blakesleeanus +1 and -1 strains were tested.

Results varied considerably (See Tables 14 &15) due to differing conditions, but were consistent in that carotene accumulation by "dark" cultures was always less than 50% of the accumulation by "light" cultures; in tests that were carried for a minimum of five days. The relative differences in carotene accumulation increased with increasing age of the cultures. The carotene level in "dark" cultures remained nearly constant after reaching its peak in three days, whereas "light" cultures continued accumulating carotene for fourteen days.

b. An additional experiment was performed with Phycomyces blakesleeanus, in which carotene accumulation by "light" versus "dark" cultures grown on media containing a range of glucose concentrations, was compared. Glucose concentrations of 10, 3.0, 1.0, and 0.30% were tested. Inoculum was +1 and -1 strains, grown singly.

As noted previously (Experiment 1b.), dry weight of mycelium decreased with decreasing glucose concentration (See Table 15). At the same time, relative carotene decreased, but not at the same rate. The decrease was

more marked in the strain +1 than in the strain -1. It appears that as the nutrient medium becomes less nutritious, the resources of the fungus are concentrated more on mycelial growth than on the perhaps non-essential synthesis of carotene.

For Mucor hiemalis, both strains, dry weight of mycelium produced by "dark" cultures is always less, by about 45%, than that produced by "light" cultures (See Table 14). This is not the case with P.blakesleeanus, both strains, for which "dark" and "light" cultures produce essentially the same dry weight under otherwise similar conditions.

4. Experiments into Effects of Reduced Oxygen Tensions

During work on "light" and "dark" cultures, it was realized that, as well as having an effect upon synthesis of carotene, light might have an effect on the maintenance of the carotene already accumulated.

Carotene is known to be labile to light and to heat. In the "light versus dark" experiments, care was taken to ensure that temperatures were equal. Though carotene may be destroyed by the incubation temperature, the destruction may be assumed to be equal for the two conditions of light and dark. However, the light factor is different, and a "light" culture may be producing a greater carotene

differential over "dark" cultures than is apparent in the results.

It may be emphasized here that all results given must be of accumulated carotene, i.e. of carotene produced less carotene destroyed. Results cannot be interpreted in the light of carotene production without a knowledge of the causes and rates of destruction in living tissue.

Experiment 10 was designed to discover if the carotene in living mycelium is destroyed by light; and if so, the degree and rate of destruction.

EXPERIMENT 10- Anaerobic transfer effect

a. Cultures of Mucor hiemalis - strain were prepared in the usual way and grown for five days to establish healthy, carotene-producing mycelium. Duplicate samples at this point showed carotene levels of about 140 micro-gm per gm dry weight.

One third of the cultures was maintained illuminated in air as a control group. The remainder were transferred to anaerobic conditions; half remaining illuminated and half into darkness. Oxygen-free nitrogen (supplied by the British Oxygen Company) was passed through the culture flasks by the method descibed earlier. A test of the influent and effluent gas with a Haldane gas analysis

apparatus showed no trace of oxygen.

The experimental cultures were kept under nitrogen for six days, with shaking to maintain them in submerged culture. Then they were returned to air to check that the mycelium was not killed by the anaerobic conditions. Two sample culture flasks were taken and analyzed every two days throughout.

b. The control cultures in air maintained a fairly constant rate of carotene accumulation up to the eleventh day, when the rate tapered off and remained very slow for the rest of the experiment (See Table 16). The experimental cultures, "light" and "dark", dropped behind slightly for the first two days after transfer to nitrogen (See Figure 5), then paralleled the controls for the next two days. This continued synthesis of carotene after transferring to anaerobic conditions is possibly a result of an accumulation of precursors while in air.

During the following two days, carotene levels in the experimental cultures kept in the dark remained fairly constant, whereas those in the light dropped markedly by about 20%. Upon return to air, the cultures in the light began to accumulate carotene again, and after a further two days had reached the level of those in the dark, which had remained fairly constant. Though several points on the graph may be in doubt, the highly suggestive low point of the "light"

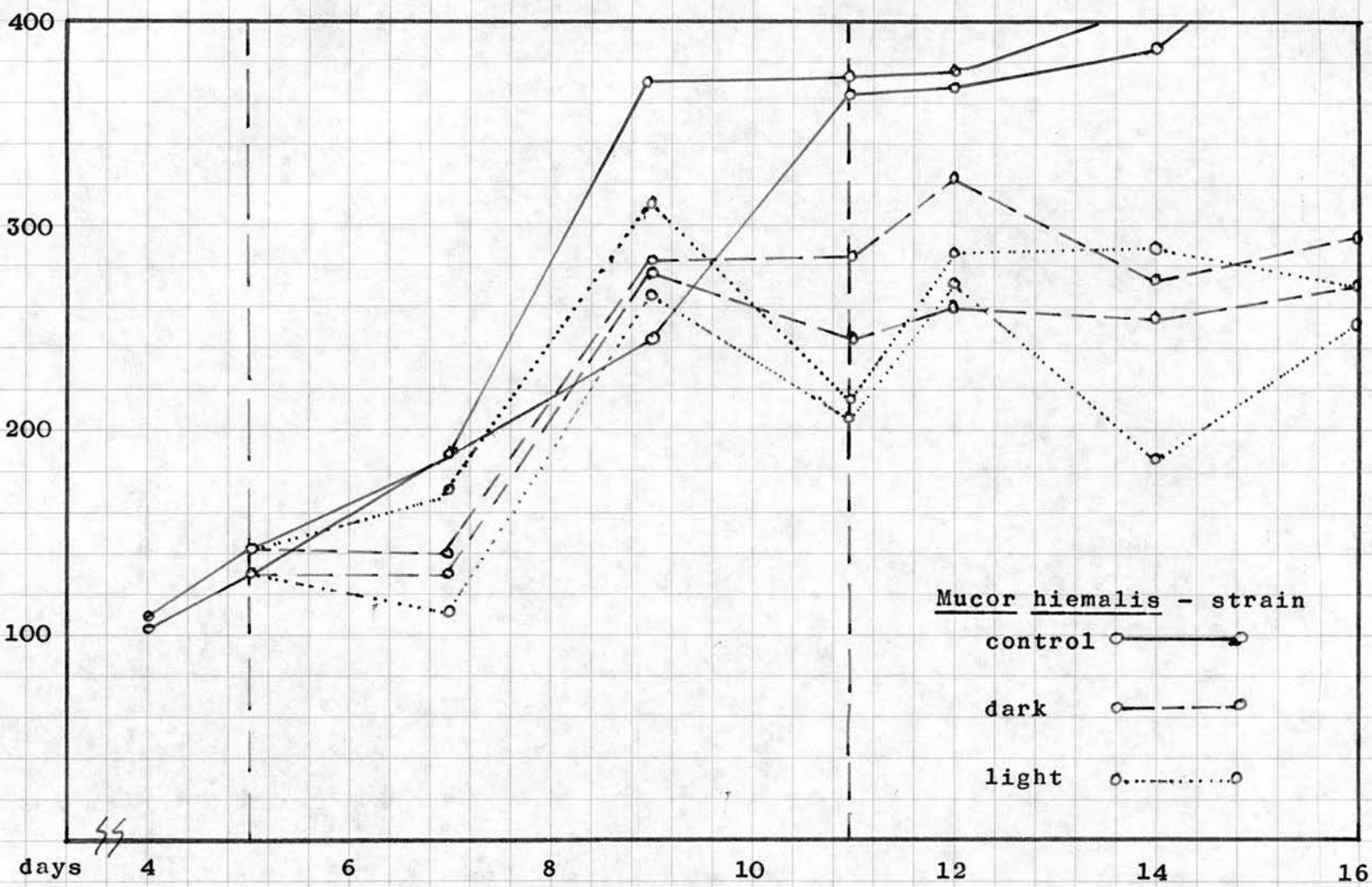


Figure 5 Effects of anaerobic transfer on carotene accumulation by Mucor hiemalis - strain, in dark and in light. Each point is a single determination. Broken vertical lines indicate time of transfer to and from anaerobic conditions.

cultures is determined with a standard deviation among the smallest.

There was little change in carotene levels for the remainder of the experiment, lasting another four days, or sixteen days in all. This experiment, though not conclusive, is certainly suggestive, and calls for repetition under conditions which are better controlled.

Goodwin states (1959) that only photosynthetic bacteria synthesize carotenoids under anaerobic conditions; and in fact decrease production of carotenoids with increasing oxygen tensions (Goodwin & Osman 1953, Cohen-Bazire etal 1957). In Phycomyces, unspecified species, it is reported (Wong 1953, cit. Mackinney & Chichester 1960) that carotenoid synthesis is unaffected by lowering oxygen tensions to 0.7% oxygen.

Experiment 11 was designed to discover the effects of reduced oxygen tensions on carotene synthesis in Mucor hiemalis, and to attempt to reproduce the results of Wong.

EXPERIMENT 11- Effects of various oxygen tensions.

a. A preliminary investigation was made, growing Phycomyces blakesleeanus -1 strain under oxygen tensions of 3% and 5% oxygen. The medium was 25 ml of liquid 5% glucose-asparagine medium, in 150 ml. culture flasks.

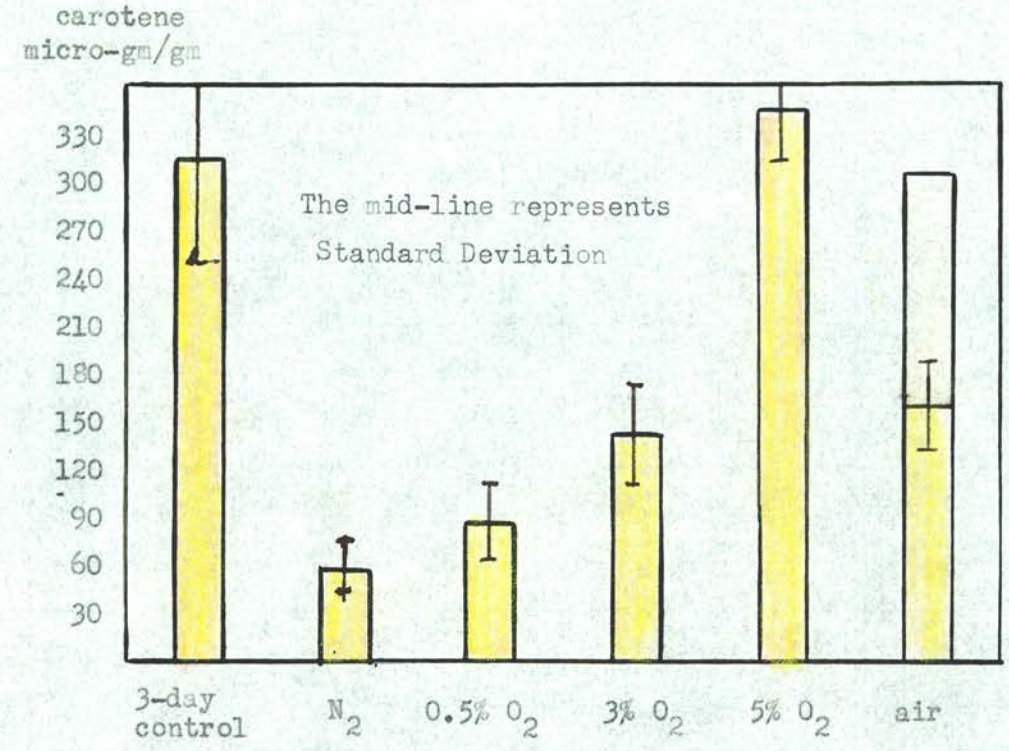
Experimental cultures were incubated at 25° C. under illumination, in submerged shake culture. All were grown in air for three days to establish healthy mycelium.

One third of the cultures was kept in air, in gauze-covered-cotton-wool plugged flasks. Nitrogen containing 5% oxygen was introduced into one half of the remaining cultures, and 3% oxygen into the other half; by the methods described earlier. The gas mixtures were provided by the British Oxygen Company.

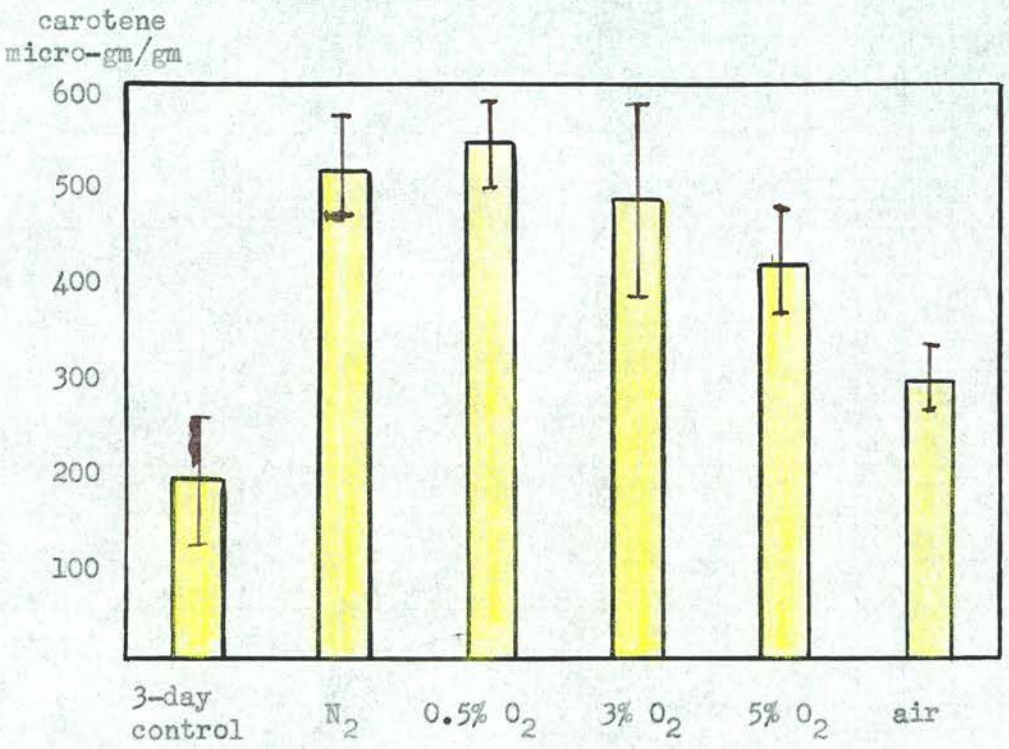
The cultures, in replicates of eight, were analyzed four days after reducing the oxygen tensions. Those in air had accumulated carotene to 174 micro-gm per gm dry weight of mycelium. Those under 5% and 3% oxygen produced and accumulated much more, to levels of 655 and 728 micro-gm per gm dry weight, respectively. (See Table 17).

There was no significant difference between the dry weights of mycelium produced by cultures under air and by those under 3% oxygen. Those under 5% oxygen produced about 20% greater dry weight.

c. On a more extensive scale, Phycomyces blakesleeanus +1 strain and Mucor hiemalis - strain were examined. Cultural conditions were the same as in the preliminary investigation. Cultures were again grown for three days before reducing the oxygen tensions. Control



Phycomyces blakesleeanus +1 strain



Mucor hiemalis - strain

Figure 6 Carotene accumulation after three days in air and four days under reduced oxygen tensions.

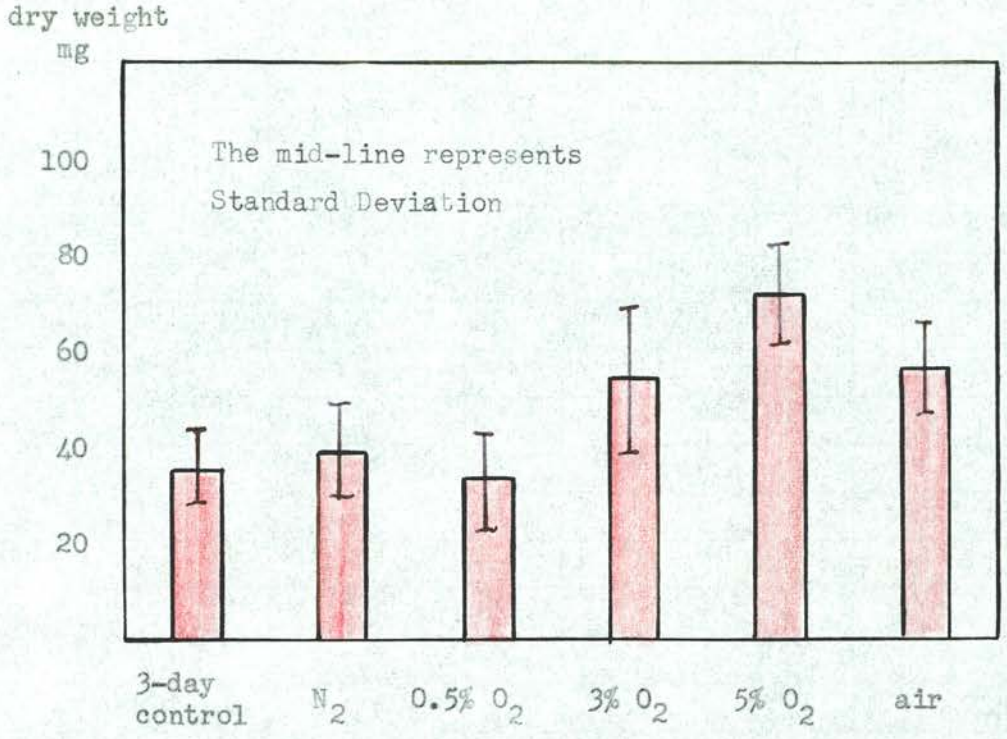
samples were taken for analysis at this stage.

The oxygen tensions tested were 20 (air control), 5, 3, 0.5, and 0 (oxygen-free nitrogen) % of oxygen. The air was introduced in the same manner as the other mixtures in order to eliminate the possibility that the cotton-wool plugs inpeded air diffusion, as in the earlier experiment. There were five replicates of each fungus at each oxygen tension.

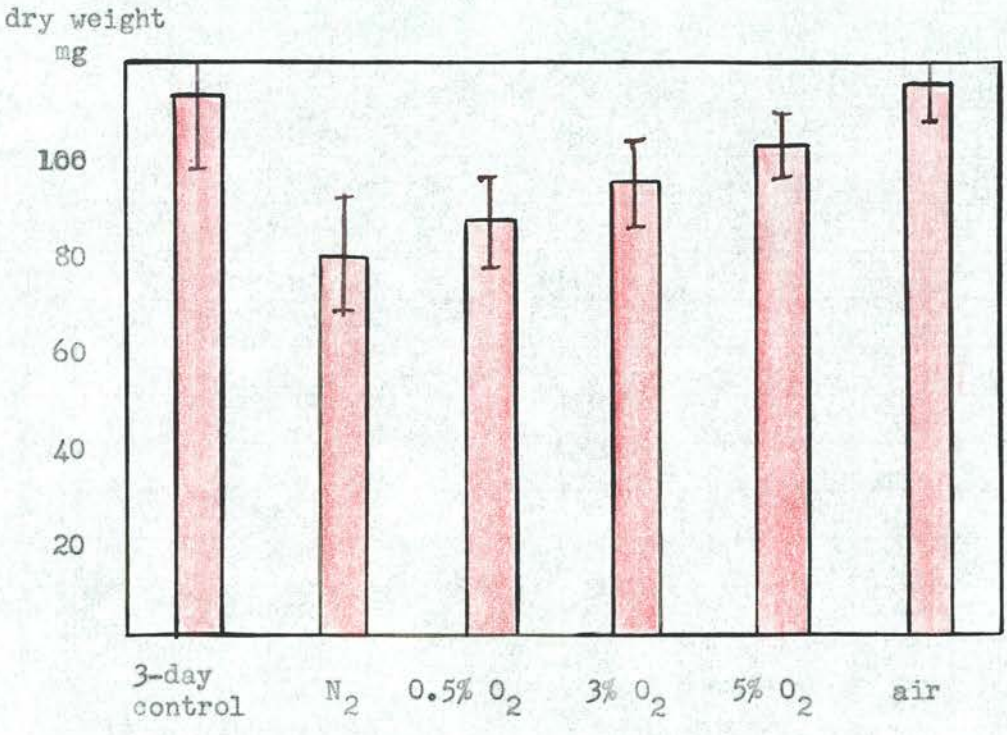
Results in general were similar to those of the preliminary investigation (See Figures 6 and 7, Table 18). Under these better conditions, Mucor hiemalis - strain showed increased micro-gm carotene per gm dry weight at all the reduced oxygen tensions, including anaerobic, over that at 20% (air). Conversely, dry weights decreased from the three-day level in all cultures except those in air.

Phycomyces blakesleeanus +1 strain, "normally" producing less carotene than the -1 strain of the same species, also showed a rather different pattern of accumulation under lowered oxygen tensions. Under 5% oxygen, the carotene level was higher than under air; under 3% oxygen it was about the same, and under 0.5% and 0% it was lower. Dry weights in no case fell significantly below the three-day level, and progressively increased to a maximum produced under air.

These results do not agree with those of Wong (1953, cit. by Mackinney & Chichester, 1960).



Phycomyces blakesleeanus +1 strain



Mucor hiemalis - strain

Figure 7 Dry weight production after three days in air and four days under reduced oxygen tensions.

Summary of Principal Results

1. The production of beta-carotene, a trace of alpha-carotene, and a colourless polyene by Mucor hiemalis and by Phycomyces blakesleeanus was confirmed.

2. Cunninghamella elegans and Absidia glauca were found to produce no carotene detectable by these techniques.

3. It was found in Mucor hiemalis and confirmed in Phycomyces blakesleeanus that carotene accumulation is very small until vegetative growth is virtually complete.

4. The maximum carotene accumulation by Mucor hiemalis is achieved after fourteen days incubation in shake culture; maximum dry weight after two days.

5. No stimulation of carotene accumulation in mixed + and - cultures was found in intra and inter-specific crosses of Phycomyces blakesleeanus, P.nitens, and Mucor hiemalis.

6. Light was found in Mucor hiemalis and confirmed in Phycomyces blakesleeanus to have a stimulatory effect upon carotene accumulation.

7. It was strongly suggested that carotene in living cultures of Mucor hiemalis is destroyed by light and remains constant in darkness under anaerobic conditions. "Light" cultures recovered upon return to aerobic conditions.

8. It was found that reduced oxygen tensions stimulation carotene accumulation in both Mucor hiemalis and Phycomyces blakesleeanus, accompanied by reduced acidity in the terminal medium.

Chapter III

GENERAL DISCUSSION OF RESULTS

GENERAL DISCUSSION OF RESULTS

The pattern of carotene accumulation in the fungi under scrutiny is not yet clear. Let us consider what is known in relation to knowledge of carotenoid synthesis in other organisms.

In the majority of carotenogenic systems, an accumulation by active synthesis of carotene does not begin until maximal vegetative growth is achieved (Goodwin 1959). This is borne out by the present results. Thus Mucor hiemalis reaches its near maximum dry weight in two days, and then begins rapidly accumulating carotene.

Phycomyces blakesleeanus does not reach maximum dry weight until after four or five days incubation. Therefore the carotene to dry weight ratio frequently appears disproportionately large, and persists so until maximum growth is achieved. This explains the actual drop in the ratio occasionally encountered after four or five days. This is also why P.blakesleeanus shows an early carotene to dry weight ratio higher than that of Mucor hiemalis of the same age, though the absolute carotene accumulation is greater in the latter.

Qualitative changes with time in carotenoid accumulation have been investigated in some algae (Goodwin 1954, Goodwin & Jamikorn 1954), to a small extent in Phycomyces blakesleeanus (Garton et.al. 1951), and in photosynthetic bacteria (Van Niel et.al. 1956). Only in the bacterium Rhodospirillum rubrum has there been discovered a major alteration, with age, in relative accumulations of various carotenoids. However, the gradual loss of beta-carotene from older cultures of Mucor hiemalis described here, and from older cultures of Phycomyces blakesleeanus (Garton et.al. 1951), may be the observable effect of a conversion into oxidized, colourless terpenes: xanthophylls. No xanthophylls have been found in analyses of these fungi, but ages of the specimens analyzed were not stated.

No previous report has been discovered of the stimulatory effect of light upon carotene accumulation by Mucor hiemalis. The effect is however reported to be common with many other fungi. Neurospora crassa accumulates coloured carotenes in the light, but colourless phytoene and phytofluene in the dark, (Sheng & Sheng 1952). The two latter compounds are named by Goodwin (1951) as precursors in the phytoene-series biosynthetic pathway for higher unsaturated carotenes.

The accumulation in the dark of colourless polyenes

found by Sheng & Sheng suggests a light-triggered reaction in the pathway. Indeed, this is found to be true in Phycomyces blakesleeanus (Chichester et.al. 1954) and in Fusarium oxysporum (Carlile 1956), where short exposures to light permitted carotene accumulation almost to the same level as accumulated under continuous illumination. Zalokar (1955) finds a similar result in Neurospora crassa.

Sheng & Sheng, by roughly correlating the action spectra of riboflavin with that of the light effect on Neurospora crassa, hint that riboflavin may be the light receptor in these reactions. This is difficult to apply generally since knowledge of the distribution of riboflavin or indeed of any flavoprotein in fungi is rather limited. The accumulation of lower members of the phytoene series suggests that it is just as possible that polyenes, possessing as they do a system of conjugated double bonds and a similar action spectrum, are the effective light receptors. Flavins have been actively proposed as the receptors in phototropic reactions (Castle 1931, Page 1956), but it does not necessarily follow that they take part in photochemical reactions.

Cantino and Horenstein (1956, 1957) find an interesting stimulatory effect of light upon growth and

carbon dioxide fixation in Blastocladiella emersonii (Cantino & Hyatt 1953); particularly in orange, carotene-containing plants. They offer a tentative hypothesis in terms of a succinate-ketoglutarate-isocitrate (SKI) cycle (Cantino & Horenstein 1956). The same effect apart from increased growth, is found to a lesser degree in colourless plants. These contain no detectable alpha, beta or gamma-carotene.

Here we have another possibility of colourless polyenes functioning as light receptors. The colourless plants, having lost the ability to complete the synthesis of carotene, altered their electron carriers to operated with less-unsaturated polyenes.

The situation regarding stimulation of carotene accumulation in mixed + and - cultures as found in the Choanephoraceae by Barnett et.al. (1956) and confirmed by Hesseltine & Anderson (1957) is still uncertain in its width of application. Clearly, such a stimulation does not occur in the members of the Mucoraceae studied. It may be stated that its presence is masked by the accumulation of various colourless polyenes. It is true that a chromatograph band was sometimes found which was colourless and fluoresced under ultra-violet light, but it is was always decidedly smaller than the smaller of

the two coloured bands; though it was itself never successfully analyzed.

Whether such a stimulation occurs in other groups remains to be discovered. At present it appears to be confined to the Choanephoraceae; and the association of carotene with sexuality remains circumstantial.

The increased carotene accumulation by Phycomyces blakesleeanus and particularly by Mucor hiemalis under reduced oxygen tensions has not been previously reported, unless such a report has been overlooked. Wong (1953, cit. Mackinney & Chichester 1960) found no effect on the fungus P.blakesleeanus, but experimental details are not stated.

In the Athiorhodaceae (photosynthetic bacteria), oxygen strongly inhibits carotenoid synthesis (Cohen-Bazire et.al 1957). It was suggested (Ibid.) that the rate of carotenoid synthesis is limited by the oxidation state of an electron acceptor and carrier. This would mean that when it is reduced, synthesis proceeds; but as it is oxidized, the rate falls.

Light also was found to inhibit carotenoid synthesis in the Athiorhodaceae. This was correlated with the hypothesis of control by the oxidation state of an electron carrier, by suggesting that the nascent oxygen resulting

from the photolysis of cell water, aids in oxidation of the electron carrier; and thus slows down carotenoid synthesis.

In the case of the fungi studied here, light has a stimulatory effect, and this cannot be reconciled with this hypothesis. An equally possible alternative may be that as the oxygen tension is reduced, terminal oxidations of the electron transport system are inhibited, causing a build-up of some of the intermediate compounds. These may be channeled, perhaps through a detoxification mechanism, into increased carotene synthesis.

The associated gradient of pH of the terminal medium in these experiments (See Table 3) supports a view of inhibited terminal oxidations. This gradient, towards a lower pH as oxygen tension is increased, is more marked in Phycomyces blakesleeanus than in Mucor hiemalis. It is possible that a greater quantity of oxidized, terminal carboxylic acids is released into the medium when the fungus is cultured in air than when it is cultured under reduced oxygen tensions.

There is at present insufficient data to offer a firm hypothesis to explain these phenomena. Still, this work outlines relatively dependable experimental and analytical techniques; and gives a general picture of

some environmental effects on carotene accumulation. Further investigations immediately suggested by the results are: spot tests of other species and groups known to produce carotene, for possible stimulation of production in mixed cultures; analysis of terminal media for oxidation products; increased oxygen tensions, and effects of carbon dioxide; Warburg manometric respiratory tests, both with oxygen and with carbon dioxide, perhaps utilizing radioactive carbon... Other ideas spring to mind. Results of these will no doubt pose fresh questions. Thus does research proceed.

APPENDIX OF TABLES

TABLE 1 Experiment 1a.

Rates of growth expressed as colony diameters in mm., of Mucor hiemalis + & -, ~~Gun~~-ninghamella elegans + & -, and Absidia glauca -; on solid media containing different glucose conc.

gluc. conc.	Age(hr) 18	26	44	65	73	97		gluc. conc.	Age(hr) 18	26	44	65	73	97	
1%	7	13	25	35	38	53	Mucor hiemalis + strain	1%	4	8	20	32	37	50	Mucor hiemalis - strain
	5	12	26	35	38	52			3	7	18	30	35	48	
2%	5	13	28	36	43	62		2%	5	10	22	35	39	57	
	6	13	30	40	46	61			5	10	24	37	40	52	
5%	6	17	31	47	54	66		5%	9	16	36	54	58	81	
	6	19	35	44	53	78			7	18	34	47	58	76	
10%	5	10	34	51	58	79		10%	5	17	30	50	58	80	
	12	22	42	55	62	76			7	17	34	48	57	72	
15%	6	12	38	46	56	76		15%	plate broken						
	8	10	27	52	60	80			6	13	32	46	58	73	
1%	9	22	28	38	43	54	C.elegans + strain	1%	11	19	24	43	48	57	C.elegans - strain
	8	12	19	28	37	57			13	22	27	42	48	58	
2%	10	23	32	40	42	57		2%	8	plate broken					
	9	19	30	42	47	59			10	18	26	46	50	61	
5%	8	18	36	52	61	79		5%	11	19	46	63	filled		
	7	18	36	52	72	f.			10	22	42	63	68	f.	
10%	12	21	42	66	filled			10%	8	17	38	54	63	f.	
	7	19	36	filled					10	18	37	54	72	f.	
15%	7	17	38	54	64	f.		15%	7	17	36	56	64	65	
	8	17	36	53	61	f.			7	18	35	56	62	f.	
1%	6	10	27	38	44	57	A.glauca -	5%	5	14	37	53	59	84	A.glauca -
	5	9	25	36	42	54		10%	7	13	34	43	44	46	
2%	5	10	29	57	62	79			6	11	36	56	58	f.	
	5	9	30	66	70	81		15%	7	13	31	42	42	50	
5%	6	12	35	69	filled				6	13	29	37	46	47	

"filled" (f.) indicates Petri plate filled to capacity; i.e. colony 100 mm. in diameter.

TABLE 2 Experiment 1b.

Dry weights (mg) of mycelium produced by Mucor hiemalis + and - strains and Phycomyces blakesleeanus +1 and -1 strains; grown for seven days in shake culture, on liquid media containing different glucose concentrations.

Glucose concs.....	0.30%	1.0%	3.0%	10%
M.hiemalis + strain	22	42	101	112
	18	55	113	138
	24	53	98	121
Means	21	50	104	124
s.d.	3	7	8	13
M.hiemalis - strain	25	94	101	110
	24	76	116	185
	29	87	113	122
Means	26	86	110	139
s.d.	3	8	9	41
P.blakesleeanus + strain	17	25	33	39
	24	21	40	40
Means	21	23	37	40
s.d.	5	3	8	1
P.blakesleeanus - strain	34	45	65	65
	30	43	63	72
Means	32	44	64	69
s.d.	3	1	1	5

"s.d." here & hereafter refers to standard deviation.

TABLE 3 Experiment 2a

Contamination of medium dispensed into dry-sterilized culture flasks.

Order of dispensing medium	1-6	7-12	13-18	19-24	25-30	
Time of heating (hours)	18	4	2	1	½	totals
Number uncontaminated	0	0	2	2	3	7
No. c. single contaminant	4	6	3	4	3	20
No.c. two contaminants	1	0	1	0	0	2
No. badly contaminated	1	0	0	0	0	1
totals	6	10	6	6	6	

From these figures it is clear that heating at 120°C. for ½ hour is sufficient for sterilization of culture flasks. The only uncontaminated media were those dispensed into flasks heated for the shorter times.

Contamination appears to take place during the dispensing of sterile medium into sterile flasks.

TABLE 4 Experiment 2b

a. Dry weights (mg) produced by Mucor hiemalis - strain in seven days in shake culture in identical media autoclaved for different times; the acid salt autoclaved separately.

Time (mins.) of autoclaving	5	10	15	30
	150	159	154	143
	163	148	152	151
	158	153	139	130
	148	160	144	137
	156	145	149	138
	161	157	159	125
Means	156	154	149	137
s.d.	6	6	7	8
s.e.	2.45	2.45	2.74	3.26

b. The same; medium autoclaved in toto.

	167	156	162	132
	152	150	137	142
	149	139	138	135
	141	158	154	123
	155	151	158	130
	152	159	157	148
Means	153	152	152	135
s.d.	8	5	11	8
s.e.	3.26	2.04	4.49	3.26

c. Significance of differences between means of media autoclaved for the same time, separately & in toto.

Difference	3	2	3	2
x	.736	.625	.572	.441
P	.46	.53	.57	.66

"s.e." refers to standard error of the mean.

"x" refers to the deviation in the normal distribution in terms of the standard deviation.

"P" refers to the probability.

All statistical calculations are based on R.A.Fisher (1945).

TABLE 5 Experiment 3a & 3b

Dry weights (mg) produced by different inoculum densities of Mucor hiemalis + and - strains.

M. hiemalis + strain

Spores in inoculum	10^8	$5X10^7$	$2.5X10^7$	10^7	10^6
	121	154	150	197	166
	154	158	179	201	121
	126	136	174	190	117
Means	134	149	168	196	135
s.d.	16	12	15	6	27

M. hiemalis - strain

Spores in inoculum	$2X10^7$	10^7	10^6	10^5
	108	109	107	64
	112	123	96	997
	101	104	122	88
Means	107	112	108	83
s.d.	6	10	13	14

TABLE 6 Experiment 4a & 4b

Dry weights and relative carotene produced by Mucor hiemalis - strain in differing culture flasks; other conditions being the same.

	Culture Flasks					
	A. 100 ml(new)		B. 100 ml(old)		C. 150 ml(N/c.)	
	dry wt mg	caro. mu.gm/gm	dry wt mg	caro. mu.gm/gm	dry wt mg	caro. mu.gm/gm
	95	324	147	289	165	319
	96	338	131	356	172	296
	85	336	136	343	159	357
	89	399	141	418	167	348
	92	354	122	367	153	405
	95	394	133	329	169	336
Means	93	358	135	350	164	343
s.d.	4	32	8	44	5	37
s.e.	1.63	13.31	3.26	17.95	2.04	15.50

Significances of differences between relative carotene:

between A & B: Dif.= 8 x = .358 P = .72
B & C: Dif.= 7 x = .735 P = .46
A & C: Dif.= 15 x = .295 P = .77

The differences between the dry weights are clearly significant; those between the relative carotenes are not.

TABLE 7 Measurements of spores from the strains of *Phycomyces* spp.

Each figure is a mean of twenty measurements.

strain	length	range	breadth	range
+1	14.4	13.3-15.8	8.8	7.5 - 10.1
-1	12.6	10.9 - 14.2	8.1	6.8 - 9.2
-6	11.6	10.9 - 13.3	7.3	6.8 - 8.4
-E45	11.7	10.1 - 13.3	7.3	6.8 - 8.4
Mean of these five	12.6	10.1 - 15.8	7.8	6.8 - 10.1
+H102	27.4	18.4 - 33.3	11.7	9.2 - 14.2

Naumov's (1939) quoted dimensions:

P. blakesleeanus Burgeff, 1925	(14)	10 - 18	8.3	
P. nitens (Agardh, 1817) Kunze, 1823	(24)	18 - 30	(11.5)	8 - 15

Gilman's (1959) quoted dimensions:

P. nitens Agardh, 1817	(23)	16 - 30	(11.5)	8 - 15

TABLE 8 Experiment 6a

Time versus carotene accumulation by Mucor hiemalis + & - strains and mixed cultures; composite results.

Age (days)	1.5	2	2.5	3	3.5	4	4.5	5	5.5
minus strain	72	85	88	134	183	198	305	357	390
	79	98	116	119	135	168	268	303	377
								348	
Means	76	92	102	122	159	183	286	336	384
s.d.	5	9	20	3	34	23	28	29	9
plus strain	128	101	99	150	137	179	117	178	160
	114	122	136	122	144	116	143	134	195
								160	
Means	121	112	118	136	141	164	130	157	178
s.d.	10	16	25	20	5	35	18	22	25
mixed culture	100	115	107	187	226	283	275	326	333
	106	110	164	241	251	269	319	387	283
								293	
Actual Means	103	113	136	214	239	276	297	335	308
s.d.	4	4	40	38	18	10	31	48	35
Theoretical (Mean +&-)	99	102	110	129	150	174	208	247	281

TABLE 8 (Continued)

Age (days)	6	6.5	7	7.5	9.5	11	12	13	14
minus strain	409	504	605	551	569	563	601	733	569
	415	521	527	574	598	608	696	611	728
	393		537		587				736
Means	406	513	556	563	585	586	643	672	678
s.d.	11	12	28	17	15	32	59	86	94
plus strain	188	213	263	280	408	494	503	500	507
	202	226	228	303	452	517	508	489	586
	179		276		434				534
Means	189	220	256	292	431	505	506	495	542
s.d.	12	9	25	17	22	17	3	7	40
mixed culture	412	394	494	468	552	417	528	468	609
	421	462	412	483	439	489	525	520	507
	393		464		481				561
Actual Means	409	428	457	476	491	453	527	494	557
s.d.	14	48	39	11	42	51	2	37	46
Theoretical (Mean +&-)	288	372	406	428	508	546	574	584	610

Age (days)	15	16	17	18	19
minus strain	536	554	422	577	458
	444	539	429	496	415
	559		536	419	
Means	513	547	462	497	437
s.d.	43	12	64	80	30

TABLE 9 Experiment 6b

Time versus dry weight production by Mucor hiemalis + & - strains and mixed cultures; composite results.

Age (days)	2	3	4	5	6	7	9.5	11	12	13	14
minus strain	113	139	165	161	161	127	129	139	144	134	144
	97	145	167	174	170	158	141	160	140	130	131
							142			144	146
Means	105	142	166	168	166	143	137	150	142	136	140
s.d.	10	4	1	9	7	23	8	15	3	7	8
plus strain	70	106	90	136	162	171	156	143	146	156	159
	61	105	156	109	201	183	146	158	154	148	151
						173	155				155
Means	66	106	113	122	182	177	152	151	150	152	155
s.d.	7	1	32	20	28	6	6	10	6	6	4
mixed culture	79	112	137	102	131	122	152	151	146	172	125
	90	75	143	116	138	128	160	156	166	138	153
							152				155
Actual Means	85	92	140	109	135	125	155	154	156	155	144
s.d.	8	22	4	10	5	4	5	4	14	22	18
Theoretical (Mean +&-)	86	124	140	145	174	160	145	150	146	144	148

Age (days)	15	16	17	18	19
minus strain	136	135	139	134	144
	138	138	141	150	136
	147		143	144	134
Means	140	137	141	143	138
s.d.	6	2	2	7	5

TABLE 10 Experiment 7a

Carotene accumulation under standard conditions by isolated strains of Mucor hiemalis + & - strains; Phycomyces blakesleeanus +1, -1, -E45, & -6 strains; and P. nitens +H102 strain.

Carotene mu.gm/gm	M.hiemalis -	M.hiemalis +	P.blakesleeanus +1	P.blakesleeanus -1	P.blakesleeanus -E45	P.nitens +H102	P.blakesleeanus -6
	605	263	206	150	146	143	69
	527	228	211	177	139	143	80
	537	276	183	123	160	149	73
Means	556	256	200	150	148	145	74
s.d.	28	25	15	27	11	4	6

TABLE 11 Experiment 7a

Dry weights of and optical densities of extracts from shake cultures of Cunninghamella elegans + & - strains and mixed cultures, varying in age.

Age (days)	3		4		5		6	
	dry wt mg	OD	dry wt mg	OD	dry wt mg	OD	dry wt mg	OD
plus strain	199	.024	225	.027	255	.093	251	.035
	207	.028	228	.033	252	.052	258	.032
					254	.019	257	.038
Means	203	.026	227	.030	254	.055	255	.035
s.d.	8	9	2	8	2	32	5	4
minus strain	177	.034	220	.029	241	.046	252	.017
	186	.022	210	.023	250	.036	249	.019
					251	.035	225	.022
Means	182	.028	215	.026	247	.039	242	.019
s.d.	6	8	7	4	8	8	16	4
mixed cultures	204	.029	230	.020	264	.049	272	.122
	207	.020	217	.030	263	.013	262	.049
					253	.008	272	.061
Means	206	.025	224	.025	260	.023	269	.077
s.d.	2	6	9	7	6	22	8	46

"OD" represents Optical Density

TABLE 11 (Continued)

Age (days)	7		13		28	
	dry wt mg	OD	dry wt mg	OD	dry wt mg	OD
plus strain	277	not read	425	.026	442	.030
	282		436	.010	439	.025
	277				443	.033
Means	279		431	.018	441	.029
s.d.	3		8	11	2	4
minus strain	264	not read	362	.040	412	.032
	267		384	.008	418	.027
	272		384		394	.022
Means	268		373	.024	408	.027
s.d.	6		16	22	14	5
mixed culture	286	not read	400	.015	423	.009
	269			.013	418	.041
	280				426	.073
Means	278		400	.014	422	.041
s.d.	9			1	4	32

TABLE 12 Experiment 7b

Carotene accumulation by intra and inter-specific crosses of Mucor hiemalis + &- strains, Phycomyces blakesleeanus +1, -1, -6, and -E45 strains; and P.nitens +Hi02 strain.

crosses..	M. hiemalis + X -	P.blakesleeanus +1 X -1	P.blakesleeanus +1 X -E45	P.blakesleeanus +1 X -6	P.blakesleeanus -1 X -E45	P.blakesleeanus -1 X -6	P.blakesleeanus -6 X -E45	P.blakesleeanus -1 X P.nitens +Hi02	P.blakesleeanus -6 X P.nitens +Hi02	P.blakesleeanus -E45 X P.nitens +Hi02
carotene mu.gm/gm	494	152	161	138	157	130	112	148	146	166
	412	185	174	116	149	108	118	130	122	140
	464	161	150	130	150	123	137	132	130	149
Actual Means	457	166	162	128	152	120	123	137	133	152
s.d.	39	15	12	11	4	12	13	10	12	13
Theoretical (Mean of the component strains)	406	175	174	137	149	112	111	148	110	147
Component strains	556	200	200	200	150	150	148	150	145	145
(See Table 10)	256	150	148	74	148	74	74	145	74	148

It will be noticed that in every case, the theoretical mean value falls within the observed mean value plus or minus twice the standard deviation.

TABLE 13 Experiment 8

Gradient of carotene and dry weight production by *Mucor hiemalis* - strain cultures at a series of distances from a light source; other conditions the same.

Distance from light source (feet)		½	1	2	4	8
dry wt mg		293	269	246	213	198
		301	252	234	205	180
	Means	297	261	240	211	189
	s.d.	11	12	8	6	13
carotene mu.gm/gm		561	458	360	291	250
		520	439	323	311	217
	Means	541	448	342	301	233
	s.d.	29	13	25	14	23

TABLE 14 Experiment 9a

Carotene production under different conditions of aeration by Mucor hiemalis + & - strains and mixed cultures; in "dark" and in "light".

a. aeration by bubbling

	Age (days)	DARK dry wt mg	DARK carotene mu.gm/gm	LIGHT dry wt mg	LIGHT carotene mu.gm/gm
minus strain	3	45	28	100	33
	4	48	42	126	189
plus strain	4	21	33	118	67
	7	26	44	150	253
mixed culture	$3\frac{1}{2}$	77	27	131	61
	4	29	26	142	181

b. shake cultures

	DARK dry wt mg	DARK carotene mu.gm/gm	LIGHT dry wt mg	LIGHT carotene mu.gm/gm
M.hiemalis - strain 7 days growth	85	69	120	169
	74	92	118	235
	82	105	119	179
	69	106	132	150
	87	104	127	159
	70	105	135	200
Means	78	97	125	182
s.d.	12	23	12	39

TABLE 15 Experiment 9a & 9b

Carotene (micro-gm/gm) production by Phycomyces blakesleeanus +1 and -1 strains in media containing different glucose concentrations; in "dark" and in "light".

glucose conc.		-1 strain "light"	-1 strain "dark"	+1 strain "light"	+1 strain "dark"
0.30%		391	135	108	50
		436	101	98	38
	Means	414	118	103	44
	s.d.	33	24	7	8
1.0%		447	372	337	102
		509	266	358	67
	Means	478	319	348	85
	s.d.	43	75	14	25
3.0%		621	283	345	147
		535	321	312	140
	Means	578	302	329	144
	s.d.	60	27	24	5
10%		754	124	502	138
		822	150	562	83
	Means	788	137	532	111
	s.d.	48	19	42	39

TABLE 16 Experiment 10_b

Carotene (mu.gm/gm) production by Mucor hiemalis - strain, "dark" and "light" cultures, transferred to anaerobic conditions and then back into air.

		anaerobic period					
Age (days)	4	5	7	9	11	12	14	16
Control (always in air, illum.)	104	141	190	243	371	374	405	498
	109	135	190	371	364	367	389	515
Means	107	138	190	307	368	371	397	507
s.d.	4	4	0	91	5	5	11	13
"dark"			136	288	242	260	275	297
			140	293	285	321	257	272
Means		(138)	138	291	264	291	266	285
s.d.			3	4	30	43	13	18
"light"			174	266	208	270	184	252
			115	314	217	285	290	272
Means		(138)	145	290	213	278	237	262
s.d.			42	34	6	11	75	14

TABLE 17 Experiment 11a

Carotene and dry weight produced by Phycomyces blakesleeanus after three days in air and a further four days under reduced oxygen tensions.

Level at 3 days		After 4 further days			
		5% O_2		3% O_2	
dry wt mg	carotene mu.gm/gm	dry wt mg	carotene mu.gm/gm	dry wt mg	carotene mu.gm/gm
43	169	54	891	35	1060
41	228	41	342	55	372
46	166	62	342	43	523
45	143	82	445	50	694
50	199	54	937	39	703
48	136	48	672	30	1120
		57	1198	33	884
		53	413	57	514
Means 46	174	56	655	43	728
s.d. 3	35	12	300	10	250

TABLE 18 Experiment 11b

Carotene and dry weight produced by Phycomyces blakesleeanus +1 strain and Mucor hiemalis -1 strain, and the pH of spent medium; upon transferring to reduced oxygen tensions.

a. Phycomyces blakesleeanus +1 strain

	3-day control (in air)	After 4 further days				
		0% O_2	0.5% O_2	3.0% O_2	5.0% O_2	20% O_2
carotene mu.gm/gm	232	67	132	80	344	144
	363	56	94	137	321	197
	332	67	79	148	389	145
	269	39	88	188	389	145
	367	67	124	163	324	166
Means	313	59	103	143	345	163
s.d.	71	10	21	36	31	25
dry wt mg.	36	43	45	79	85	100
	33	37	30	35	67	101
	36	46	41	46	73	107
	40	22	30	54	64	101
	36	47	17	60		
Means	36	39	32	55	72	102
s.d.	5	9	10	15	7	6
pH	3.97	4.12	3.72	3.68	3.69	3.51
	3.95	4.22	4.08	3.69	3.57	3.58
	3.99	4.14	3.98	3.75	3.78	3.73
	4.00	4.00	3.79	3.67	3.77	3.82
	3.98	4.04	3.76	3.70		
Means	3.98	4.11	3.86	3.70	3.70	3.66
s.d.	2	9	16	3	10	13

b. Mucor hiemalis -1 strain

	3-day control (in air)	After 4 further days				
		0% O_2	0.5% O_2	3.0% O_2	5.0% O_2	20% O_2
carotene mu.gm/gm	210	466	572	316	473	284
	194	510	536	323	343	312
	190	568	508	508	405	295
	212	526	588	574	397	310
	169	523	512	654	438	283
Means	195	519	543	477	411	297
s.d.	17	37	35	132	52	14
dry wt mg.	128	87	87	106	101	116
	117	76	72	98	107	110
	94	67	86	90	100	114
	90	64	85	93	101	121
	130	79	99	90	96	126
Means	112	79	86	95	101	117
s.d.	19	10	10	7	4	6
pH	3.54	3.47	3.50	3.38	3.50	3.53
	3.53	3.44	3.47	3.51	3.41	3.50
	3.88	3.45	3.40	3.45	3.48	3.60
	3.58	3.48	3.29	3.37	3.40	3.50
	3.77	3.57	3.32	3.50	3.58	3.20
Means	3.66	3.48	3.40	3.44	3.47	3.41
s.d.	15	5	9	6	7	16

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