

THE INFLUENCE OF CARBON DIOXIDE
ON GROWTH AND METABOLISM OF
CHAETOMIUM GLOBOSUM.

A THESIS

presented by

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in part fulfilment of the requirements for the

Ph.D. Degree

of the

University of London

July 1964.

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ABSTRACT

An investigation has been undertaken into the influence of carbon dioxide on growth and metabolism of Chaetomium globosum.

Removal of CO₂ from their environment did not adversely affect the rate of germination of the spores; in fact a slight stimulation was observed. Tests showed that this effect was not due to carry over of possible "germination stimulants" from the parent culture in the spore suspension fluid. However, no further growth of the resultant sporelings occurred when CO₂ was withheld from their environment. The sporelings developed a "granular" appearance after 4 days, by which stage no protoplast could be demonstrated by staining methods, and they were no longer viable when transferred to normal atmospheric environment.

This type of "stunted" growth, typical of growth in the absence of CO₂, could not be reproduced by omission from the medium of one or more of the components, or by using a purer type of agar, in the presence of CO₂. None of a wide range of chemical compounds tested was able to replace CO₂ in promoting normal development of the mycelium. The only substances found capable of permitting growth in the presence of a CO₂ absorbent, were natural extracts.

Fixation of CO₂ by the fungus was demonstrated using ¹⁴CO₂. Products of fixation included purines, pyrimidines and amino acids. Inclusion of jute extract in the medium during fixation had little effect on fixation, but inclusion of aspartic acid in the medium depressed fixation by 70%.

The relationship between carbon dioxide and growth and metabolism of Chaetomium globosum is discussed, and an attempt has been made to assess the results in the light of current knowledge.

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INTRODUCTION

The fact that carbon dioxide may have a notable effect on the growth and development of microorganisms was recorded in some of the earliest systematic investigations into the environmental requirements of bacteria and fungi. Possibly the earliest observation in this connection was that of Pasteur and Joubert (1; 1877) who stated that Bacillus anthracis was "killed" by CO₂; their paper contains no details of the concentration of CO₂ used, nor of the time needed for "killing" the bacteria. This statement was soon disputed by Szpilmann (2; 1880) and by Grossman and Mayerhausen (3; 1881); and a more extensive study by Buchner (4; 1895) showed that bacteria showed very different degrees of susceptibility to the gas. However, the most remarkable discovery in these early days was that of Vinogradsky (5; 1890) who showed clearly that the CO₂ effect was not one of mere stimulation at some concentrations and inhibition at others, but that the presence of the gas was absolutely essential as a source of carbon for certain nitrifying bacteria. Scattered observations are recorded in the literature during the succeeding twenty years, and in general it

was found that although high concentrations of CO₂ were often inhibitory, small amounts were stimulatory and possibly essential.

Interest in the question seems to have increased from about 1918, in which year Wherry and Ervin (6; 1918) showed that CO₂ was essential for survival of the tubercle bacillus, and the next ten years saw the publication of two series of papers, the first by Rockwell and Highberger (7 - 11; 1921-1927) and the second by Valley and Rettger (12 - 15; 1925-1930). (A useful review of work from 1877 to 1928 is to be found in: Valley, Quart. Rev. Biol. 3, 207 1928 (16)). Rockwell and Highberger confirmed the observations of Wherry and Ervin, and noted also that complete removal of CO₂ from the environment inhibited the growth of many bacteria, including Proteus vulgaris, Pseudomonas pyocyanea, Staphylococcus albus and Bacillus coli (Escherichia coli).

The work of Valley and Rettger was very comprehensive, and their studies covered over one hundred different bacteria. In their earliest paper (12) they pointed out that under ordinary cultural conditions bacteria are frequently exposed to

an atmosphere containing some 1% of CO₂, and they showed that "complete" removal of CO₂ for 24 - 40 hours killed some species (P. vulgaris, Vibrio cholera, B. typhosum and Lactobacillus acidophilus) and inhibited the growth of many others. A need for CO₂ was demonstrated for 82 organisms (13, 14), and interesting variations in the requirements of different bacteria were noted. Many benefited by the presence of CO₂ up to concentrations of approx. 1%; in some instances different strains of the same organism (e.g. P. vulgaris) needed different concentrations of CO₂ for optimum growth. Again, some organisms failed to grow when all atmospheric CO₂ was removed (e.g. B. subtilis), while others needed also the removal of bicarbonate from the medium (e.g. B. cereus). The same authors (14) found indications that some anaerobes needed CO₂. Where CO₂ was found to have a harmful effect (in higher concentrations), this was thought to be due to a pH effect rather than to any direct effect of the gas itself.

In a similar connection, Winslow, Walker and Sutermeister (17; 1932) ascribed the lag period in bacterial growth (in some cases) to the need for

the CO₂ concentration in the environment to reach a critical minimum before fully active growth could occur, and suggested that the extension of the lag period which often results from aeration was due to the removal of CO₂ (cf. also Whitehead, Jones and Robertson, 18; 1958).

Other instances of the need for CO₂ for bacterial growth have also been recorded and it is of particular interest to note that not only the vegetative phases of growth may be affected. As was first clearly demonstrated by Wynne and Foster (19; 1948) germination of the spores of some anaerobes (Clostridium botulinum) may be delayed indefinitely under otherwise suitable conditions by the exclusion of CO₂. (An indication of this fact was found by Morrison and Rettger (20; 1930) in their studies on spore germination.) However, Wynne and Foster were unable to delay germination of three other Clostridia, (Cl. chauvei, Cl. perfringens and Cl. histolyticum), or of four aerobic spore-formers, (B. brevis, B. megaterium, B. mesentericus and B. subtilis), by withholding CO₂ and they suggested that the media used already contained some factor which "by-passed" CO₂ in the metabolic pathway.

Microorganisms other than bacteria have been found to be susceptible to the influence of CO_2 , and in many cases to have an absolute requirement for it, both for vegetative growth and for other phases of development. Lopriore (21; 1895) found that the germination of spores of Mucor mucedo was retarded in the presence of abnormally high concentrations (greater than 10%) of the gas, and that under these conditions the mycelium formed "swollen cells". Brown (22; 1922) in his very extensive survey of fungal nutrition noted that many saprophytic fungi were inhibited by the presence of excess CO_2 , and that the relative degree of inhibition was more marked at lower temperatures. Durrell (23; 1924) demonstrated the need for CO_2 for the germination of spores of Basisporum gallarum. Platz, Durrell and Howe (24; 1927) observed that the germination of spores of Ustilago zeae was stimulated in the presence of respiring plant tissue (e.g. orange) and concluded that respiratory CO_2 was the responsible agent. In this instance, ^{the optimum} concentration of CO_2 was approximately 15%, and they considered that the effect arose from a "definite action" of the gas - that is, participation

in some chemical reaction, and not merely through some pH effect.

In the same year, Rippel and Bortels (25; 1927) recorded "poor germination" of spores of Aspergillus niger in an atmosphere deprived of CO₂. Little interest seems to have been taken in the effect of CO₂ on fungi until late in the 1940's, but since then a considerable amount of research has been devoted to the matter; and since the advent of isotopic tracer techniques more detailed studies of the chemical pathways, both in fungi and bacteria, have been pursued.

Yanagita (26; 1957) studied the effect of CO₂ on the germination of spores of A. niger and was able to confirm the results of Rippel and Bortels as regards inhibition of swelling and sprouting of spores, when the culture was aerated with CO₂-free air. However, when the experiment was repeated in static culture in CO₂-free air about 40% germination occurred. This he attributed to build up of respiratory CO₂, since the spore density was high (7×10^6 per ml.). He found the optimum CO₂ concentration for germination to be 0.1% (v/v) and complete inhibition at approximately 10% (v/v).

Vakil et al (27; 1961) attempted to overcome the discrepancies encountered by Yanagita by using a lower spore density (1000 - 2000 per ml.) in surface culture. Absence of CO₂ delayed germination of spores for 12 hours, after which they concluded that respiratory CO₂ had built up to a level capable of initiation of germination. Under their experimental conditions, they found the optimum concentration of CO₂ for germination to be 0.5% and total inhibition at 3%.

Before describing the suggested chemical mechanisms through which CO₂ may act, brief reference may be made to the effects on growth and morphogenesis which are particularly striking with some fungi. In 1948 Emerson and Cantino (28) published the first of a series of papers in which the latter author and his colleagues have recorded some observations on members of the genus Blastocladiella. B. emersonii, considered to be a "simple" aquatic fungus, was found to be sensitive to low concentrations of CO₂ (HCO₃⁻ in solution) in whose presence certain obvious morphological features were completely

modified. A population of spores usually develops into mature "plants" of three types:- (i) thin wall and colourless (OC), comprising the bulk (more than 90%) of the population, (ii) orange coloured (O) plants containing much carotene and (iii) resistant sporangial plants (RS) having a thick wall impregnated with melanin. In an atmosphere of 99.5% CO₂, or in the presence of HCO₃⁻ in solution (10⁻²M), the OC population is largely replaced by RS plants. The unusually high incidence of RS types which is found in thickly growing cultures was ascribed to a local increase in CO₂ concentration. (Cantino 29: 1956). Evidently in this organism CO₂ plays a key role in morphogenesis, though whether it is indispensable for growth as such is not certain. However, another member of the genus, B. britannica, is entirely insensitive to CO₂ - "Even total lack of atmospheric CO₂ had no detectable effect on B. britannica." (Horenstein & Cantino; 30; 1961).

Bright (31; 1949) refers to an "adverse effect" of an increased concentration CO₂ on sporulation of yeast, but gives no quantitative data; on the other hand Bettelheim and Gay (32; 1963) were able to reduce or delay the formation of ascospores

by depriving the yeast of CO₂. Possibly an optimum concentration of CO₂ exists.

Gitterman and Knight (33; 1952) demonstrated the necessity of CO₂ for growth of Penicillium chrysogenum, and Taber and Vining (34; 1957) showed a similar requirement for Claviceps purpurea.

Gunderson (35; 1961) found that the growth of Fomes annosus, a fungus parasitising the interior of roots and trunks of trees, was stimulated by a slight increase in the partial pressure of CO₂.

Barnett and Lilly (36; 1955) found that accumulation of CO₂ **within closed** vessels prevented the sporulation of Choanephra cucurbitarum (Mucorales).

An effect of CO₂ on the fruiting of Collybia veluptipes was studied by Plunkett (37; 1956).

In a stagnant atmosphere which would allow an accumulation of CO₂, this fungus forms only rudimentary pilei, and in controlled experiments it was shown that an increase in the concentration of CO₂ from atmospheric (0.03%) to 1% produced a significant decrease in cap diameter.

Newcombe (38; 1960) studied the response of Fusarium oxysporum f. cubense to CO₂ and found that very few chlamydospores were produced; the gas

overcame the fungistatic action of some soils, and allowed spores to germinate in otherwise unfavourable conditions.

Lones and Peacock (~~38a~~; 1960) studied the conditions which lead to the formation of the "spherule" as opposed to the mycelial form of Coccidioides immitis, and found that this transformation was encouraged by the use of a heavy inoculum, and also, very notably, by exposure to an atmosphere containing 10% CO₂.

Working with Mucor rouxii, Bartnicki-Garcia and Nickerson (38b; 1962) found that incubation of the fungus under anaerobic atmosphere of relatively high pCO₂ (0.3atm. or more) produced a drastic shift in vegetative morphogenesis from the usual filamentous mycelium to a yeast-like form composed of spherical budding cells.

Some important work has been done during recent years by Barinova (39 - 43; 1953 - 62); unfortunately it has not been possible to consult the original papers, and it has been necessary therefore to rely on the published abstracts only. She showed that CO₂ stimulated the growth of Aspergillus niger, A. flavus, Rhizopus nigricans and P. chrysogenum,

particularly in the earliest stages of growth, and in formation of conidia, but that the stimulation was in some cases dependent upon the pH of the medium rather than on a direct effect of CO₂. She found that the growth of A. niger in a glucose medium was stimulated in the presence of certain di- and tri-carboxylic acids but only when CO₂ was also present; the gas was found also to reverse the inhibition caused by malonic and mesotartaric acids. She studied the effect of CO₂ on the respiration of A. niger, and its nitrogen-phosphorus utilisation. Her ideas concerning the role of CO₂ will be discussed later.

Finally, Moss (44; 1961) was able clearly to show that CO₂ was essential for the production of perithecia by Chaetomium globosum under certain conditions. He grew the fungus on a Czapek-Dox agar medium containing 0.5% glucose, and by the fifth day after inoculation, when the colony diameter had reached some 20-25mm., and perithecial initials had begun to appear, he removed small discs (about 5mm. diameter) from near the centre of the plates and transferred these to Petri dishes containing water or some nutrient solution. At this stage, the supply

of glucose was virtually exhausted from the medium present in the dissected disc, so that Moss was able to study the effect of various substances on perithecial development without interference from residual glucose. He found that by rigidly excluding CO_2 from his discs, perithecial growth was almost completely prevented, except when succinic acid (the only one of several substances tried) was present.

This outline is sufficient to indicate how important is the role, in anabolic pathways, of CO_2 -- a compound which, from the earliest days of physiological chemistry has been looked upon as a product of catabolic processes. Its anabolic role was largely ignored long after it had been recognised, but when some attention finally became focussed on this aspect, it was natural that attempts should be made to define the nature of its activity with more precision. Two main approaches have been made to this problem. In the first, efforts have been made to replace CO_2 by other substances, -- those into which CO_2 might conceivably have been incorporated; in the second, use has been made of isotopic carbon tracing, firstly to demonstrate that

CO₂ was indeed fixed by the organism and secondly to follow its path after fixation.

Many substances have been tested in "replacement" experiments, but attention has been directed more particularly to two groups, namely various carboxylic acids associated with the Krebs cycle, and certain N. compounds, including purines and naturally occurring amino acids. Since the Krebs cycle involves a number of decarboxylations, it is natural that the members of the cycle should receive especial attention.

These attempts at replacement have met with rather scanty success, and no uniform picture emerges from them. Lwoff and Monod (45; 1946, 46; 1947) found that, for E. coli, glutamic acid and certain other C₄ and C₅ acids could stimulate growth at low tensions of CO₂, but were without effect at the lowest attainable tensions of the gas (cf. Barinova). Ajl and Werkman (47; 1948, 48; 1949), however, claimed that glutamic acid and a number of other amino acids as well as some, but not all, of the Krebs cycle acids could replace CO₂ completely for their strain of E. coli. Gerhardt and Wilson (49; 1950) were unable to demonstrate replacement of CO₂ by glutamic acid or by a number of C₄ and C₅ acids

for growth of Brucella abortus.

It had long been recognised that most strains of Neisseria meningitidis benefit by an increased CO₂ supply (Gates 50; 1919), and Tuttle and Scherp (51; 1952) were able partly, but not wholly, to replace CO₂ by small amounts (0.03% in the medium) of guanine plus uracil plus cytosine, especially if casein hydrolysate and a mixture of vitamins B were also present. Even more effective was 0.1% yeast extract, which was apparently able to replace CO₂ entirely under the experimental conditions, but attempts to isolate any specific factor from yeast extract were unsuccessful.

Gitterman and Knight (33) made an extensive study of the CO₂ requirements of P. chrysogenum, and found that oxaloacetic acid alone among the Krebs cycle acids could replace CO₂. None of the amino acids tested was effective, nor was casein hydrolysate, yeast extract, an extract of the mycelium of P. chrysogenum, or culture filtrates from the mould. Corn steep liquor was, however, fully effective.

For producing sporulation of B. sphaericus, α -oxoglutaric acid was effective (Powell and Hunter,

52; 1955), while an entirely different type of compound, namely oleic acid, was claimed by Diebel and Niven (53; 1955) to replace CO₂ for Lactobacillus leichmanii. Moss's observation on the effect of succinic acid on sporulation of C. globosum has already been mentioned. Certain sugar phosphates have been shown to encourage perithecial growth of C. globosum (Buston, Jabbar and Etheridge, 54; 1953) but these were unable to replace CO₂.

More recently, Lowe and Evans (55; 1962) failed to obtain a response with 0.1% yeast extract, some Krebs cycle acids, aspartic acid, 3 phosphoglyceric acid or carbamyl phosphate in the case of Rhizobium japonicum, one of five Rhizobium spp. which were found to require CO₂.

A somewhat different approach to the "replacement" problem was used by McLean and Purdie (56; 1955). In this procedure, the atmosphere was enriched with ¹⁴C CO₂ and the amount of ¹⁴C fixation was noted under standard culture conditions. Various substances which could possibly substitute for CO₂ were incorporated into the medium and any decrease in fixation was observed; if the addition of any compound resulted in a lowering of the CO₂ uptake,

that compound was considered to be a member of the metabolic sequence for which CO_2 was essential. In experiments on Serratia marcescens, McLean and Purdie found that the fixation of CO_2 fell to as little as 6% of the "control" in the presence of a combination of purines, arginine and asparagine, and that this decrease was greater than the sum of the decreases produced by the individual components of the mixture. Asparagine could be replaced by certain of the Krebs cycle acids. It is to be noted that in no experiment was the fixation of $^{14}\text{CO}_2$ reduced to zero.

Perhaps the most categorical statement on this subject is that of Ajl and Werkman (48) who stated that, for Aerobacter aerogenes and E. coli, all compounds replacing CO_2 do so by "yielding keto-acids which in turn by amination, transamination or other reactions serve as substituents for CO_2 ." These keto-acids were considered to be members of the Krebs cycle or their metabolic precursors, especially oxaloacetic and α -ketoglutaric acids. This suggestion, although plausible and possibly true for this organism, would seem an over-simplification of the position.

It can only be said that from all this work, it seems that, for some organisms, CO_2 may be partly replaced by some (but by no means all) of the Krebs cycle acids, by certain purines or pyrimidines, and by some aminoacids; or by combinations of these compounds. It may also be replaced by some complex mixtures (i.e. corn steep liquor) of unknown composition, but in some instances no replacement has been found effective, and no clear picture of the way in which CO_2 acts has been forthcoming.

Chemical mechanisms by which CO_2 becomes incorporated into metabolic pathways have been proposed on many occasions since the first demonstration by Wood and Werkman (57; 1936) of non-photosynthetic fixation of CO_2 by heterotrophic bacteria, and almost all the suggestions have had their experimental basis in isotope tracer studies using $^{14}\text{CO}_2$. It has become evident that not only is the capacity to "fix" CO_2 found in a very large range of very diverse organisms and tissues, but also that several different mechanisms exist, involving carbohydrates, non-nitrogenous acids, amino acids, purines and pyrimidines and possibly other types of compound. It is not necessary here to describe the details of photosynthetic fixation, but it may be emphasised

that even in this the actual mechanism by which CO_2 is incorporated is a "dark" reaction - the incorporation of CO_2 into ribulose diphosphate - which is found also in non-photosynthetic bacteria. In this system the key enzyme is ribulose diphosphate carboxylase, and the earliest recognisable produce of CO_2 fixation is 3-phosphoglyceric acid, followed rapidly by a number of phosphate esters associated with the Embden-Meyerhof glycolysis pathway. The same enzyme has been demonstrated in Thiobacillus spp. (Santer and Vishniac, 58; 1955; Vishniac, Horecker and Ochoa, 59; 1957; Susuki and Werkman, 60; 1958). In Hydrogenomonas spp. the enzyme is to some extent adaptive; it appears under autotrophic conditions but is almost entirely absent under heterotrophic conditions e.g. in the presence of lactate or other organic source of carbon. Another instance in which ribulose diphosphate carboxylase is formed inductively is that of E.coli grown on a xylose medium in the presence of CO_2 ; in the absence of CO_2 no enzyme can be detected (Vishniac et al.; 59). Quite recently, Nicholas and Rao (61; 1964) have shown that Nitrosomonas europaea, grown in a medium containing $\text{Na}_2^{14}\text{CO}_3$ at 30°C . produces labelled phosphoglyceric

acid, phosphoenolpyruvate, various phosphate esters and the amino acids serine and glycine within 30 minutes. This pattern is identical with that found in green plants. It appears that the ribulose diphosphate-phosphoglyceric acid-hexose mechanism is typical of photosynthetic and chemosynthetic systems, which differ only in the means by which the "reducing power" necessary to reduce phosphoglyceric acid is mobilised.

The other major pathway for the incorporation of CO_2 , typical of many, if not all, heterotrophic organisms, results in the formation of certain keto-acids, particularly those which participate in the Krebs tricarboxylic acid cycle. The details of the steps leading to the keto-acids differ from organism to organism, and in some cases the first detectable product may be an amino acid rather than a keto-acid, but this latter fact may usually be explained by an immediate amination or transamination of the keto-acid initially formed.

Several mechanisms by which actual fixation of CO_2 into keto-acids or related compounds may occur have been identified. Wood and Werkman reported the early appearance of succinic acid when CO_2 is

taken up by Propionibacterium spp., and discussed possible pathways to account for this observation (57; 62; 1936), but reached no definite conclusion as to the nature of the first product of fixation. However, Wood, Werkman, Hemingway and Nier (63; 1941) obtained evidence that pyruvic acid participated in the fixation process, and more detailed schemes to account for the observed facts were put forward by Delwiche, Phares, Carson, Long and Berger (64; 1956).

A system which is able, in the presence of ADP, to fix $^{14}\text{CO}_2$ to give oxaloacetic acid labelled at the β -carboxyl atom was discovered by Utter and Wood (65; 1946) in crude pigeon liver extracts; here the acceptor molecule was phosphoenolpyruvate. A corresponding enzyme system was found in yeast (Cannata and Stoppani, 66; 1959). Another system based upon phosphoenolpyruvic acid has been found in certain chemoautotrophic bacteria by Suzuki and Werkman (67; 1957), and is perhaps the same as that reported by Herbert (68; 1957) in Micrococcus lysodeikticus. This fixes CO_2 into phosphoenolpyruvate with the loss of inorganic phosphate, and does not require ADP as a phosphate acceptor; oxaloacetate is again the product. This reaction is virtually irreversible and can proceed at low CO_2 tensions.

Anderson and Ordal (69; 1961) showed that in a CO_2 -dependent fermentation of glucose by Cytophaga succinicans, $^{14}\text{CO}_2$ was fixed in one carboxyl group of succinic acid derived from PEP via oxaloacetic acid. Allied systems have been identified in some fungi also. Woronick and Johnson (70; 1960) demonstrated the presence of an ADP-dependent carboxylation of PEP in cell-free extracts of A. niger. A second pathway was also discovered by Woronick and Johnson (70) in A. niger, namely one in which CO_2 condensed directly with pyruvic acid (i.e. not via PEP) in the presence of ATP; ADP and inorganic phosphate resulted, and the $^{14}\text{CO}_2$ appeared in the β -carboxyl group of oxaloacetic acid.

Possibly the best known carboxylations are those activated by reduced pyridine nucleotides (particularly NADPH). This group includes systems activated by malic dehydrogenase, isocitric dehydrogenase and 6-phosphogluconic dehydrogenase. The malic enzyme is widely distributed in plants and is also found in the Lactobacilli, in which it is NADH dependent. This enzyme is responsible for a reductive synthesis of malate from pyruvate and CO_2 , without the intermediate appearance of oxaloacetate (at least

attempts to detect this acid have failed); and although the equilibrium is far in favour of decarboxylation, it can in some cases be shifted in the reverse direction.

Isocitric dehydrogenases, both NADH and NADPH dependent, have been detected in microorganisms. They closely resemble the malic enzyme, and catalyse a reaction in which α -ketoglutarate and isocitrate participate; and again, the possible intermediate, namely oxalosuccinate, is not detectable, and the balance is normally in favour of decarboxylation.

Reversible decarboxylation of ribulose-5-phosphate to 6-phosphogluconic acid in the presence of NADPH has been demonstrated in plants, animals and microorganisms. A net synthesis of 6-phosphogluconic acid from ribulose-5-phosphate and CO_2 has been shown by coupling the carboxylation with a system generating a continuous supply of NADPH. The enzyme from yeast has been partially purified (Horecker and Smyrnotis, 71; 1952), and both NADH and NADPH linked systems have been reported by Hochster (72; 1957) in Aspergillus spp.

Another decarboxylation, first demonstrated in pig heart and later in Mycobacteria involves ATP and biotin, in whose presence CO_2 is fixed into

propionyl CoA to give methylmalonyl CoA, which then isomerises in the presence of vitamin B12 to give succinyl CoA. It has been suggested that this mechanism (via methylmalonyl derivatives) is concerned with the synthesis of those branched chain fatty acids typical of Mycobacteria.

Tracer studies have often indicated that various amino acids become strongly labelled after relatively brief exposure to $^{14}\text{CO}_2$, although, as has already been suggested, they may in actual fact be secondary products derived from the keto-acids whose origin has just been discussed. The pattern of labelling is of considerable interest, since it may give an indication of the identity of the immediate precursors of the amino acids. Aspartic acid, glutamic acid, arginine and serine have all been found among the amino acids which become labelled in the early stages. Ehrensvärd (73; 1948) found early labelling in Torulopsis utilis of aspartic acid, glutamic acid and arginine, while Marr and Wilson (74; 1951) reported that of the total ^{14}C fixed from $^{14}\text{CO}_2$ in B. abortus, 40% was found in the carboxyl group of glycine. In a medium containing no preformed amino acids, alanine was also labelled.

There seems little in common between the findings of Ehrensvärd and of Marr and Wilson, and the conclusion must be that more than one pathway of CO₂ fixation exists. Some more detailed work was carried out by Abelson, Bolton and Aldous (75, 76, 77; 1952) who used E. coli for their experiments. Of the tracer ¹⁴C used, over 50% was accounted for in some 16 different compounds, mainly the amino acids of bacterial proteins and the bases of nucleic acids. Of the amino acids, 6 contained, in sum, 95% of the total label entering the proteins; and of these, aspartic acid was by far the most strongly labelled, followed by arginine, threonine and lysine; glutamic acid and proline were only weakly labelled. More detailed investigation showed that the ¹⁴C was incorporated into the β -COOH group of threonine and lysine, the β -COOH group of aspartic acid and the amidine group of arginine. These authors sought further information on the subject by modifying their culture media by adding the unlabelled amino acids. In general, this resulted in a decrease (up to 90%) of the amount of ¹⁴C entering the amino acids. When aspartic and glutamic acids were present in the medium, the labelling of these and of all the

other amino acids was suppressed; other amino acids, when added suppressed only the labelling of the same amino acid, although citrulline suppressed the labelling of arginine. These observations led to the suggestion that aspartic and glutamic acids were precursors of all the others, and that arginine was formed by a pathway similar to (or identical with) the Krebs-Henseleit mechanism. In the third paper, (77), it was shown that ^{14}C enters the purines and pyrimidines by separate pathways, and that in neither case do amino acids play any intermediate role. Summing up their work, these authors conclude that at least five different pathways can be traced in E. coli, namely those giving rise to (1) purines, (2) pyrimidines, (3) aspartic acid, (4) glutamic acid and (5) the amidine group of arginine. The studies of these authors were extended by a group of American workers, (Roberts et al, 78; 1957) in a very comprehensive study of biosynthesis by E. coli. In their experiments they were able to account for almost all the ^{14}C incorporated by this organism, and further, they had firm evidence concerning a number of metabolic pathways. They standardised certain routine analytical procedures, and since these

have been adopted to some extent in the present work it is appropriate to summarise them at this point. After exposure to $^{14}\text{CO}_2$, the cells were harvested and subjected to the following successive extractions:- cold 5% trichloroacetic acid; 75% ethanol; 75% ethanol + ether; hot 5% trichloroacetic acid. The first extract contains low molecular weight water-soluble material, consisting largely of transient metabolites, some of which were not identified. Fraction two, (ethanol + ethanol-ether) contains lipid and lipoprotein. The third fraction consists largely of nucleic acids, whilst the residue after extraction as above comprises the bulk (90%) of the cell proteins. Amino acids, purines and pyrimidines, nucleic acids and proteins all received the label, and these workers concluded that there were not less than four independent pathways along which $^{14}\text{CO}_2$ travelled, namely, one leading to the purines; another to the pyrimidines; a third to guanidine group of arginine; and a fourth, given in more detail, leading via aspartic and glutamic acids to ornithine, lysine, proline, threonine and other amino acids. Since all the common acids concerned were closely related to the Krebs cycle, these authors concluded that this cycle represented the major

mechanism of incorporation of CO_2 .

In 1958, Noble, Reed and Wang (79; 1958) studied the CO_2 uptake by P. digitatum, and again noted the early presence of ^{14}C in aspartic and glutamic acids - within 30 minutes from the moment of exposure to $^{14}\text{CO}_2$. These amino acids were followed by proline, threonine, lysine, isoleucine and tyrosine; alanine and other amino acids related to pyruvic acid were not labelled, nor, (curiously since tyrosine was labelled), was phenyl alanine. The presence of glucose in the medium may have accounted for the production of sufficient amounts (unlabelled) of these amino acids.

A rather different picture was obtained by Tepper and Wilson (80; 1958), who worked with Brucella abortus. As in the earlier work, (Marr and Wilson, 74), the exceptionally heavy labelling of glycine was noted and of the total amount of ^{14}C in this amino acid, 90% was present in the carboxyl group. However, it seemed that this very considerable incorporation into glycine might be less important than was first thought. Arginine, aspartic acid and glutamic acid (in that order) also became labelled, and the relative proportion of ^{14}C in glycine diminished markedly as the concentration

of $^{14}\text{CO}_2$ available was lessened.

An important contribution was made by Stoppani, Conches, de Favelukes and Sacerdote (81; 1958) in their detailed study of the assimilation of CO_2 by Saccharomyces cerevisiae and S. carlsbergensis. They showed that S. cerevisiae fixes CO_2 during the aerobic metabolism of glucose, pyruvate, acetaldehyde and acetate, and during the anaerobic phases (glycolysis) of glucose breakdown. This fixation was but little affected by fairly wide differences in pH (pH 3.1 - pH 7.0). The use of $^{14}\text{CO}_2$ showed that fixation occurred in the Krebs acids, in compounds related to glycolysis and in a number of amino acids, with aspartic, glutamic, malic and the tricarboxylic acids as the main reservoirs, although the details of the pattern varied somewhat according to the nature of the substrate oxidised. They noted that, for S. cerevisiae anaerobic conditions led to an increased proportion of the ^{14}C label in the dicarboxylic acids (succinic, fumaric and malic) and that the pattern for S. carlsbergensis was always of this type. The same pattern was found for S. cerevisiae when oxidative mechanisms were inhibited by Antimycin A.

They considered that assimilation of CO_2 took place via oxaloacetate, resulting from carboxylation of phosphoenolpyruvate.

The importance of aspartic acid was again observed by Wright (82; 1960), who found that in Streptococcus bovis, ^{14}C derived from $^{14}\text{CO}_2$ enters chiefly this amino acid, with lesser amounts in threonine, glutamic acid and the nucleotides. He concluded that CO_2 fixation reactions are the principal source of aspartic acid in this organism, since ^{14}C -aspartic acid supplied in the medium was not taken up to any extent.

Bartnicki-Garcia and Nickerson (83; 1962) found aspartic acid as one of the major CO_2 fixation products of the yeast-like form of Mucor rouxii. The relative proportions of ^{14}C fixed into the three amino acids which become labelled after 30 minutes was aspartic acid, 75.1% threonine, 14.5% and glutamic acid 10.4%. Fixation into nucleic acids was also detected.

Apart from what may be classed as "Krebs cycle pathways" of fixation, a few other mechanisms have been reported or suggested. Pine and Barker (84; 1954), working with Butyribacterium rettgeri,

found that acetate became labelled in both carbon atoms; they did not formulate any pathway to account for this fact, but they considered that no di- or tri-carboxylic acid cycle mechanism was involved, nor was a pathway via serine and glycine.

Pine (85; 1956) reported that during fermentation of glucose in the presence of $^{14}\text{CO}_2$ by certain strains of Actinomyces, the ^{14}C entered both the methylene and the carboxyl carbons of succinic acid, but later, (86; 1960) this statement was modified and the presence of ^{14}C in the methylene carbons was denied.

Another mechanism has been suggested by Seliber (87; 1957). He has claimed that oxalate and formate may play a part in CO_2 fixation; but this suggestion seems not to have been followed up.

An unusual pathway of CO_2 fixation has been suggested by Kusumose et al. (88; 1959), who have shown that cell free extracts of Mycobacterium avium contain an enzyme system capable of carboxylating acetyl phosphate to give malonate or a derivative thereof; the presence of Co A and certain metal ions was essential. The significance of this observation is not yet clear, but there may be a link (via malonate) with lipid synthesis.

Finally, mention must be made of some extremely interesting observations by Charles (89; 1962) on the nutritional needs of certain Neurospora mutants. The particular mutants in question were one requiring preformed arginine and one certain pyrimidines, and it was found that by increasing the quantity of CO₂ in the culture-atmosphere (up to 30% v/v), the mutants were able to grow in the absence of arginine and pyrimidines respectively. In a later paper, Charles and Broadbent (90; 1964) reported that at least 10 Neurospora mutants were "CO₂-mutants", and in the same paper they discussed the possible biochemical background of this observation.

In a few instances, suggestions have been made concerning the precise role of CO₂ in stimulating (or initiating) some particular phase of development. Thus Powell and Hunter (52), who had concluded from their experiments with B. sphaericus that sporulation in this organism depended upon (1) the presence of an intracellular factor and (2) an

external stimulus which could only act effectively if (1) had reached a critical level, suggested that CO_2 was concerned in the production of the intracellular factor through a mechanism involving dipipicollic acid. A somewhat related pathway was considered also by Gollakota and Halvorson (91; 1960).

Possibly the most detailed discussions of the role of CO_2 in morphogenesis are those of Cantino and his colleagues (summarised in Cantino, 92; 1961). Mention has already been made (p. 7) of the effect of CO_2 in producing a morphogenetic change in Blastocladiella emersonii whereby the "OC" type of plant is replaced by the "RS" type. This transformation can be prevented by removal of HCO_3^- , but only in the earlier stages of development; after approximately 43% of the generation time has lapsed, the development of "RS" plants is not halted by removal of bicarbonate from the medium.

Cantino's explanation is that bicarbonate interferes with the Krebs cycle at one or more of the steps involving decarboxylation, thus causing an accumulation of keto-acids or their immediate precursors which are then diverted by shunt mechanisms promoted by newly induced enzymes into alternative

synthetic pathways. The idea that new enzymes appear is supported by (1) a de novo appearance of melanin, of γ -carotene, of a polyphenol oxidase system and of a new electrophoretically-separable protein; (2) the disappearance of succinoxidase, of cytochrome oxidase and of α -keto-glutaric oxidase; (3) the rapid synthesis of isocitrate in bicarbonate media, contrasted with the lack of isocitrate in media deprived of CO_2 ; and (4) a sharp change in enzyme pattern coinciding with the "point of no return" (i.e. approximately 43% of generation time) in the presence of bicarbonate. These suggestions imply that the Krebs cycle is in a state of delicate balance and that its pattern may easily be drastically altered by an alteration in the concentration of any of its members, or by the presence of extracyclic metabolites related thereto. This view is shared by Roberts et al. (78) who noted that the addition of (inter alia) aspartic or glutamic acid immediately changed the flow pattern of the cycle.

This hypothesis does furnish the beginnings of a reasonable chemical explanation of the effect of small amounts of CO_2 on morphogenesis in this fungus; whether similar changes of metabolic pattern could

be established elsewhere has yet to be proved, but as mentioned above (p. 8), no such system seems to exist in the related fungus, B. britannica.

SCOPE OF THE PRESENT WORK

The work here recorded developed from the observations of Moss (44) on the influence of CO_2 on the production of perithecia by Chaetomium globosum. Moss carried out a few experiments with $^{14}\text{CO}_2$, and showed that there was a definite fixation of the gas into the mycelial tissues, but he did not attempt any studies of the distribution of the isotopic carbon among the various components of the tissue. This question has now been studied in some detail, and the pattern of incorporation has been found; this constitutes the second section of the thesis. In the first, details are given of some observations on the influence of CO_2 and other compounds on the various phases of growth of C. globosum - e.g. spore germination and vegetative growth. These observations are complementary to those of Moss on perithecial development, and together with the latter give a fairly complete picture of the qualitative effects of CO_2 on the life cycle of this fungus.

EXPERIMENTAL

MATERIALS AND METHODS

a) Materials

The organism used throughout these studies was the same strain of Chaetomium globosum Kunze as that originally isolated by Basu (93; 1947). It was maintained by subculturing on Czapek-Dox agar slopes enriched with aqueous extract of jute.

The basal medium contained, per litre, NaNO_3 , 2.0 g., KH_2PO_4 , 1.0 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g., KCl , 0.5 g., $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g. and glucose, 5.0 g. For solid media, 10 g. of Oxoid No. 3 agar were added. All media were sterilised at 10 lb. for 15 minutes, except where noted. Incubation was at 30°C except where noted. During the course of this work, it became evident that the organism had lost some of its vigour in that the production of perithecia and percentage germination of spores were lower. Therefore a fresh subculture was made from Basu's original isolate.

The aqueous extract of jute was prepared by boiling powdered jute (Corchorus capsularis)

with distilled water for 30 minutes. For every 10 g. jute powder, 100 ml. distilled water were allowed, and after boiling, the jute powder was filtered off and the filtrate made up to the original volume. The extract was used with an equal volume of double strength basal medium, giving a final medium which contained the extract of 5 g. of jute in every 100 ml.

b) Preparation of inocula

For inoculation, both spore and vegetative inocula were used.

1) Spore inocula

A spore suspension in sterile distilled water was prepared from a three week old slope. The suspension was then centrifuged and washed twice with sterile distilled water before resuspending in sufficient fresh sterile distilled water to give a count of 30 - 40 spores per microscope field when a drop was examined on a slide under the low power (x 60). This was the inoculum used in the majority of experiments; in some experiments the number of washings in sterile distilled was varied.

2) Vegetative inocula

For some of the radioactive-tracer experiments, a vegetative inoculum was used. 4 - 5 day old mycelium was harvested and washed once with normal saline. It was then homogenized for 10 seconds in the Waring Blendor in sterile distilled water such that 100 ml. water contained 100-150 ml. dry weight mycelium. A known volume (5 ml.) of this suspension was inoculated into an equal volume double strength medium for use.

c) Measurement of growth and sporulation

Growth of the fungus in liquid culture was estimated from the dry weight of the mycelium after filtering, washing and drying overnight at 98-100°C.

Growth on solid media was estimated by measuring the diameter of the colony in two directions at right angles and taking the mean, although this method was inadequate to reflect differences in type of colony. Dry weights of colonies on agar were measured by freeing the colony from the agar in boiling water, washing with hot water, filtering and drying as above. Where perithecia were present, their dry weight could be measured by holding the cultures at -23°C overnight, when the perithecia could be scraped off fairly free of mycelium and dried and weighed as above. The dry weight of the mycelium

was then obtained as usual.

d) Carbon dioxide fixation experiments: fractionation

Procedure

This was a modification of that used by Roberts et al. (78) with E. coli. The scheme is shown diagrammatically below:-

- | | | |
|--|----------------------|--------------------------------------|
| 1. Mycelium washed with 0.9% saline soln. | | normally contained no radioactivity: |
| Centrifuged | <u>supernatant</u> → | discarded. |
| 2. Residue + 5 ml. 25% ethanol held in ice bath 0°C for 30 mins. | | <u>"25% Ethanol Fraction"</u> . |
| Centrifuged | <u>supernatant</u> → | |
| 3. Residue + 5 ml. 75% ethanol held in water bath 40-50°C for 30 mins. | | <u>"75% Ethanol Fraction"</u> . |
| Centrifuged | <u>supernatant</u> → | |
| 4. Residue + 5 ml. 75% ethanol/ether as above, 40-50°C for 30 mins. | | <u>"Ethanol/Ether Fraction"</u> . |
| Centrifuged | <u>supernatant</u> → | |

5. Residue + 5 ml. 5%
trichloroacetic acid
held in boiling water
bath for 60 mins.

Centrifuged	<u>supernatant</u> →	<u>"Hot T.C.A. Fraction"</u>
6. Residue	→	<u>"Protein Fraction"</u> .

e) Analysis of fractions

1. 25% Ethanol, 75% Ethanol and Ethanol-Ether Fractions

The measurement of radioactivity in these fractions was carried out directly and their further analysis not performed.

2. Hot Trichloroacetic Acid Fraction

The trichloroacetic acid was removed by ether extraction before assaying the radioactivity of the fraction. It was then hydrolysed at 100°C on a boiling water bath for 30 mins. with 0.1% hydrochloric acid before concentration in vacuo and chromatography.

3. Protein Fraction

The residue left after extraction was hydrolysed in 6N hydrochloric acid at 108°C for 4 hours in a sealed Pyrex tube. Residual hydrochloric acid

was removed by reduction to dryness three times in vacuo. The hydrolysate was taken up in 50% ethanol, assayed for radioactivity and chromatographed.

f) Chromatography

1. Apparatus

Chromatography, both one and two dimensional, was routinely carried out in a Smith 10" Universal Chromotank (Shandon Scientific Co. Ltd.) equipped for ascending chromatograms. The apparatus was designed to accommodate five 10" square papers, enabling five 2-dimensional or up to 50 1-dimensional chromatograms to be run simultaneously.

Chromatography of jute extract was performed in a cylindrical glass tank cm. high and cm. in diameter fitted both for ascending and descending chromatograms.

2. Solvents

1.	<u>Solvent</u>	<u>Composition</u>	<u>Application</u>
	n Butanol	100 ml.	First dimension for amino acids and protein hydrolysates.
	Formic acid	25 ml.	
	Water	100 ml.	
2.	Phenol	80 gm.	Second dimension for amino acids and protein hydrolysates.
	Conc. NH_4OH	0.3 ml.	
	Water	20 ml.	

3.	Tert. butanol	70 ml.	Nucleic acid
	12N HCl	6.7 ml.	hydrolysates (One
	Water	20 ml.	dimensional.)
4.	Methanol	80 ml.	Jute extract
	Formic acid	15 ml.	chromatography.
	Water	5 ml.	

3. Detection of compounds after chromatography

1) Amino acids and proteins

The chromatograms were sprayed with 0.1% ninhydrin in distilled water saturated with n-butanol and developed by heating at 105°C for 5 minutes, when the amino acids gave typical purple, blue or yellow spots.

2) Purines and pyrimidines

These compounds were detected by viewing the chromatograms under the U.V. light, when guanine gave a purple fluorescence and the others gave dark areas due to absorption of the light.

4. Authoradiography

Radioactive compounds on chromatograms were detected by exposing the chromatograms to Kodak "KodiRex" X-ray film for varying time periods, usually in the region of two - three weeks. The chromatograms were stapled to the film and stored in Kodak X-ray exposure holders during exposure.

g) Assay of radioactivity

0.10 ml. aliquots of solutions to be assayed for radioactivity were dried onto $\frac{3}{4}$ " diameter metal discs, which had previously been treated with 0.1% Teepol in 50% ethanol and allowed to dry to ensure even distribution of the radioactive material on the discs. The discs were placed on the second shelf of a lead castle containing a Mullard MX 123 end window Geiger-Mueller tube linked to an Ecko N 530 E Automatic Sclaer. Corrections were applied to the recorded radioactivity for background radioactivity and where applicable for counts lost due to the 400 μ sec. dead time of the GM. tube.

RESULTSPART 1 SOME EFFECTS OF CARBON DIOXIDE ON PHASES OF
GROWTH OF CHAETOMIUM GLOBOSUM.A. Germination of spores and development of sporelings

In any consideration of the events which occur when a fungal spore commences active growth, one difficulty is to decide upon some criterion by which the initiation of germination may be judged.

The first result of exposing a spore to an environment in which growth will result is, presumably, that a physical absorption of water occurs, owing to the hydrophilic-colloidal nature of the spore contents. This is evidenced by the fact that contact with water is followed very quickly by a change of appearance of the spore, namely a swelling of the spore accompanied by the appearance of one or more vacuoles within it. If no more than this has happened, it might be supposed that removal of the spore to a drier environment would simply result in a reversal of the absorption process and the spore would remain in the resting state, while still retaining its viability. There seems little doubt, however, that irreversible changes quickly begin to occur, although no outward evidence of these can be seen, and it is when the first irreversible change takes place that germination can

be said to commence. It may be some time - possibly a matter of hours - before visible evidence of germination, such as the emergence of a germ tube, can be found; but before this, essential changes in the internal organisation of the spore must have occurred.

Some evidence has been obtained which shows that the irreversible stage of germination is rapidly reached. A suspension in distilled water, containing 0.001% Tween 80 as a wetting agent, was prepared from spores obtained by a modification of the method of Yanagita (26;1957), which enabled spores to be harvested dry, thus reducing the time necessary for the production of a spore suspension. After 15, 30 and 90 minutes, portions of the suspension were used as inocula in the usual manner on Czapek-Dox-glucose agar. At the same times, the spores were centrifuged down from other portions of the suspension, dried in vacuo, (approximately 15 minutes), resuspended and similarly used as inocula. All spore suspensions which had been in contact with water and dried gave lower percentage germination than the controls, and the longer the period of contact with water, the lower the germination (Table 1). The most obvious explanation would be that the suggested irreversible change in the great

Table 1.

The effect of drying spores, which had been held in aqueous suspension, on their subsequent germination.

Time of removal of sample from suspension (mins)	% Germination (6hrs.)	
	Control.	Dried.
15	45	16
30	48	13
90	-	5

majority of the spores within 90 minutes. There is a possibility that drying the spores in vacuo leads to the loss of some volatile stimulant of germination; but in this case, all samples of spores, taken at different times, would have been affected equally by the drying process, and a progressive diminution of percentage germination would be unlikely.

The nature of these changes has been discussed by Gottlieb (94;1964), who mentions, inter alia, changes in lipid distribution, synthesis of ribonucleic acid and protein, development of enzymic activities, and increase in oxygen uptake prior to the emergence of the germ tube. To quote Gottlieb, "Germination can no longer be defined by morphological changes alone; it must also be related

to functional changes by which a spore is transformed from a dormant state of low metabolic activity, to one of high activity. Formation of the germ tube is the outward and visible sign that this metabolic change is complete." From a simple practical standpoint however, it seemed reasonable to take as a criterion of germination the first visible emergence of a germ tube.

Bearing in mind these points, detailed observations were made on the earliest stages of germination of spores, on one hand under ordinary atmospheric

conditions, and also under conditions in which CO_2 was excluded as completely as possible from the medium and the environment. In the course of experiments in this direction, it was found that germination was to an appreciable extent affected by factors other than the presence of CO_2 - age of the spores from which the suspension was prepared, dilution of the inoculum, etc. Some of these factors were studied more closely, and will be described later; but a standard procedure was adopted, in which the " CO_2 free" and the "control" experiments were performed in a manner which allowed a true comparison of the two sets of observations. The routine procedure ensured that all spore suspensions used in a group of experiments were prepared from spores of the same age; that a standard procedure for preparing the suspensions was followed and that the spore count of the inocula was always the same, or was known. Further, in following the development of spores and sporelings, a method of marking the microscope field was in some cases adopted, whereby it was possible to follow the changes taking place in a particular group of sporelings during the course of development. Usually, small Petri dishes (4.5 cm.) were cemented onto microscope slides and

within a larger Petri dish (15 cm.) in which was placed 25 ml. 5% sodium hydroxide when CO₂-free conditions were to be studied. Each large dish accommodated three of the smaller ones, and in these the cultures were grown.

Cultures were removed for observation (of the selected, marked field desired) after 2, 4, 6 24 and 48 hours in the case of the controls, and also after 5 - 12 days in the case of the CO₂-free cultures. Two cultures were examined after 2 hours; these were then returned to the culture dish and re-examined after the intervals stated above; at the second (4 hour) examination, two additional cultures were examined. Similarly, at 6 hours, the two original (2 hour) cultures, the two 4 hour cultures and two new 6 hour cultures were examined; and so on throughout the course of the experiment. In practice, this routine meant that all cultures taken for examination were exposed to the air, and hence to CO₂, during the time that the examination was being made. This was immaterial in the case of the control plates; but evidently, once the CO₂-free plates had been exposed, even briefly, to the air the possibility (or even probability) existed that sufficient CO₂ might have been absorbed to "trigger off" or to allow growth to proceed (see below p. 59).

Table 2 indicates the results of a typical series of such tests, and indicates the percentage of spores which had germinated at the time stated - germination being judged solely by the appearance or otherwise of a germ tube visible under the microscope ($\times 100$) (see fig. 3). These results show a number of interesting features.

In the first place, the maximum percentage of spores which germinated was frequently low, i.e. of the order of 30 - 40% within 48 hours; and other occasions it was as high as 70% in 8 hours. A more interesting feature of these results was that they failed to show any significant effect of the deprivation of CO_2 on the ability of the spores to develop a germ tube under the conditions in which the tests were made. This is apparent from the figures given in Tables 2&3, and the corresponding curves (Figs. 1 & 2). Indeed, from this experiment it could be concluded that the removal of CO_2 (to the extent permitted by the experimental conditions) tended to increase the percentage germination, although from the slope of the curve (Fig. 2) the maximum rate of germination was slightly higher in the presence of atmospheric CO_2 .

TABLE 2

Comparison of percentage germination of spores on Czapek-Dox-glucose agar in atmospheres (a) containing CO₂ and (b) deprived of CO₂. I. Spores derived from original culture (see text).

Time (hours after inoculation)	2	4	6	24	48
Percentage spores germinated + CO ₂	0	5	12	23	(29)*
Percentage spores germinated - CO ₂	0	5.5	15	28	30

* the growth of mycelium made accurate detection of ungerminated spores difficult.

TABLE 3

Comparison of percentage germination of spores on Czapek-Dox-glucose agar in atmospheres (a) containing CO₂ and (b) deprived of CO₂. II. Spores derived from fresh subculture of isolate.

Time (hours after inoculation)	2	3	4	5	6	7	8
Percentage spores germinated + CO ₂	0	2	27	43	61	66	70
Percentage spores germinated - CO ₂	0	18	33	45	61	74	79

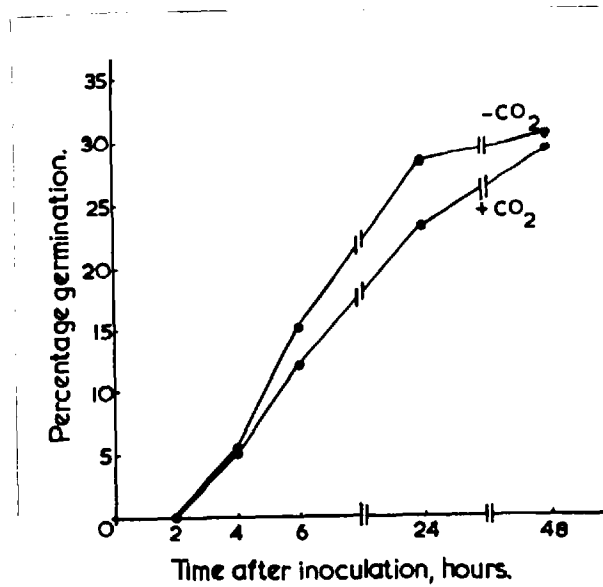


Fig. 1. Graph of germination of spores in atmospheres a) containing CO₂ and b) deprived of CO₂ against time. (Original culture)

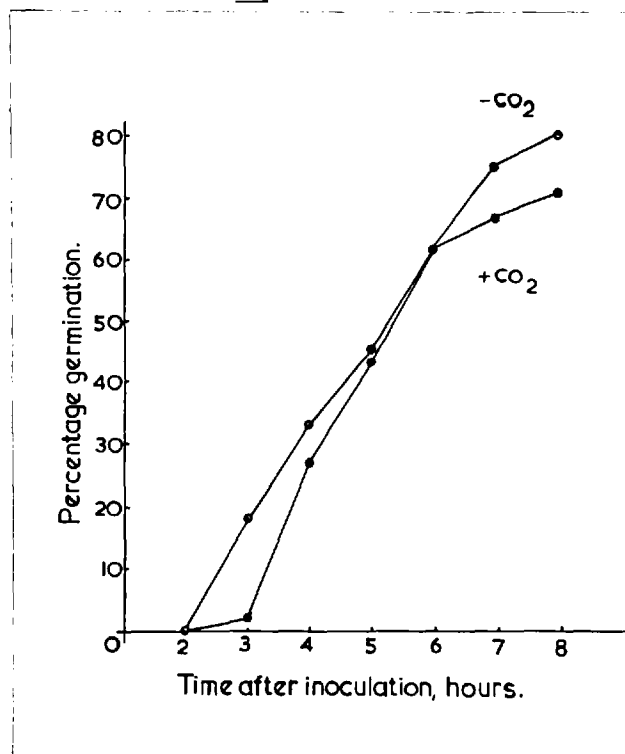
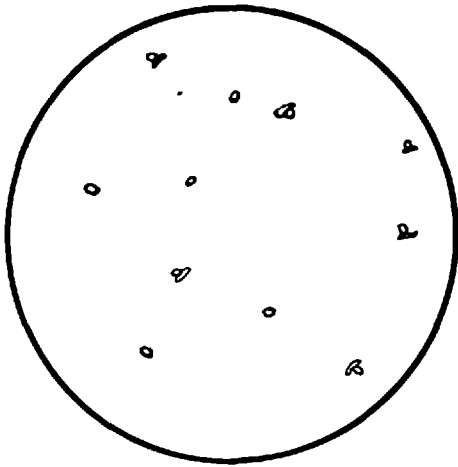
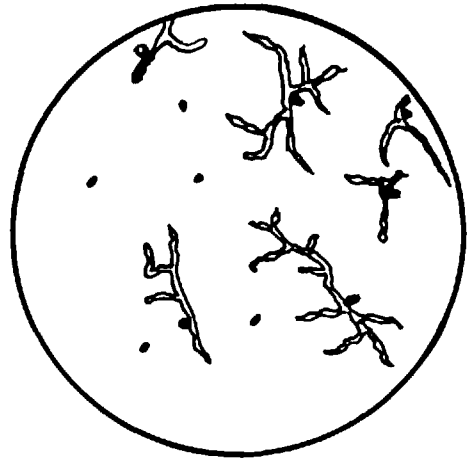


Fig. 2. Graph of germination of spores in atmospheres a) containing and b) deprived of CO₂ against time. (Fresh culture)

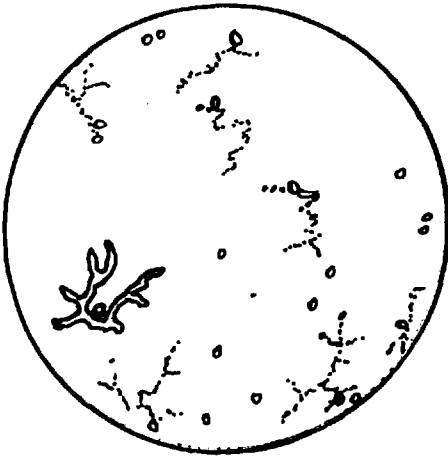


a. 6 hours.

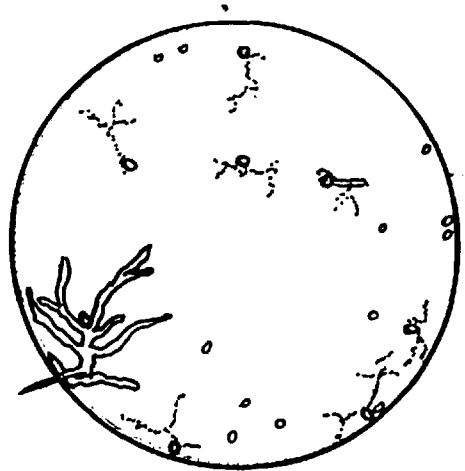


b. 12 hours.

Fig. 3. Growth of sporelings held in unrestricted atmosphere. (x150).

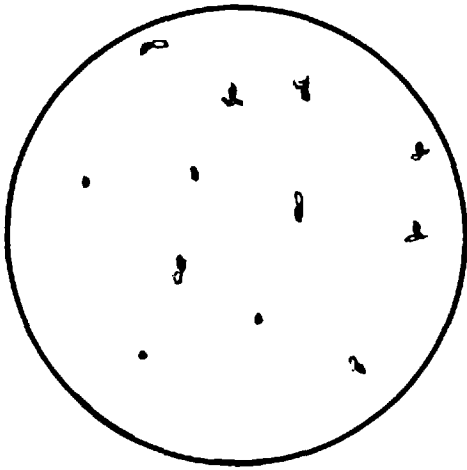


a. 8 days.

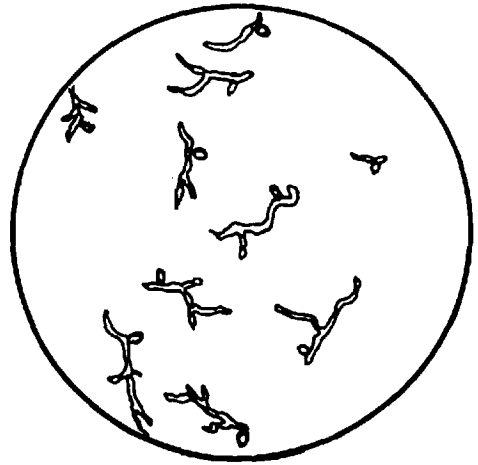


b. 9 days.

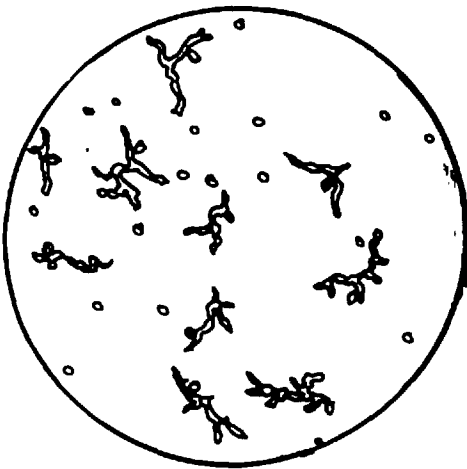
Fig. 6. Abnormal growth of an isolated sporeling after 8 days in a CO₂ restricted atmosphere. (x150).



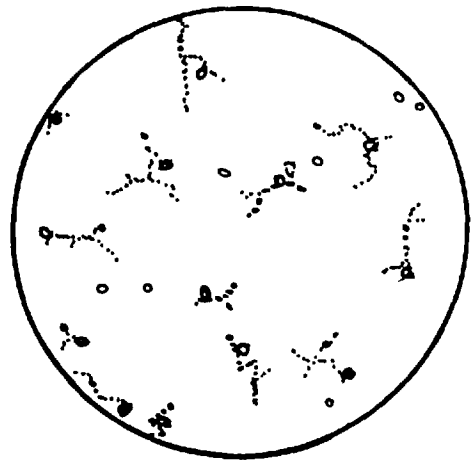
a. 6 hours.



b. 24 hours.



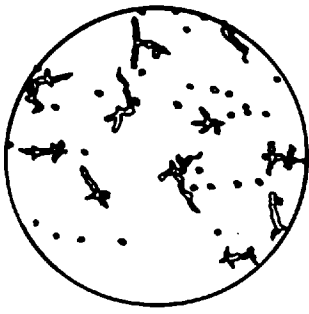
c. 48 hours.



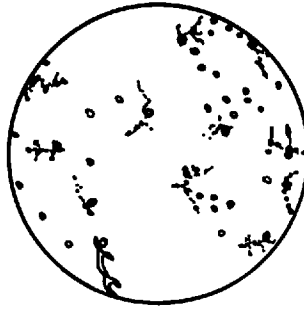
d. 5 days.

Fig. 4. Growth of sporelings held in a CO_2 restricted atmosphere.

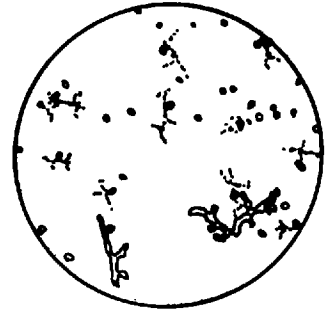
(x150).



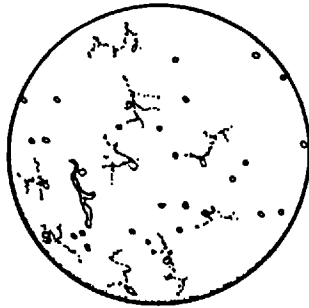
a. 2 days.



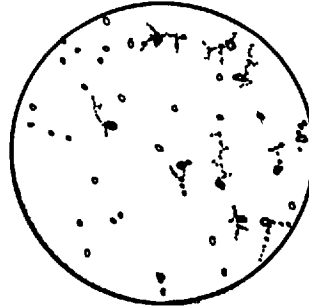
b. 4 days.



c. 5 days.



d. 6 days.



e. 8 days.

Fig. 5. Growth of sporelings held in a CO_2 restricted atmosphere.
(x100).

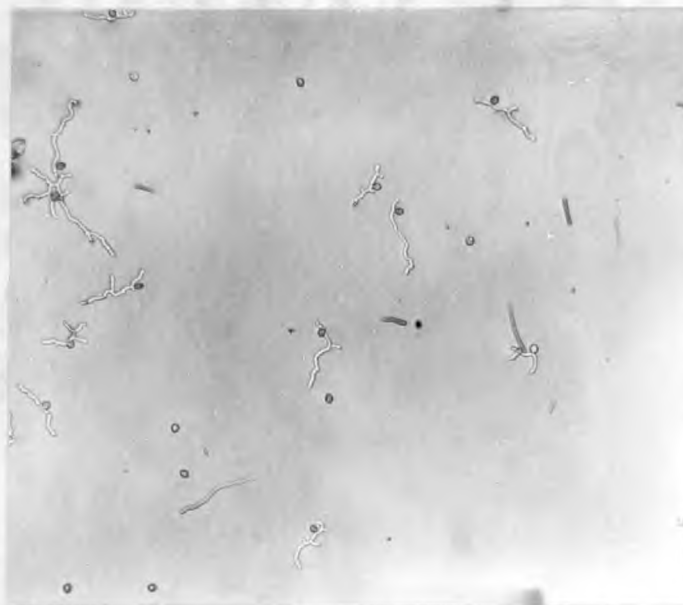


Fig. 7. Growth of sporelings deprived of CO_2 , 24 hours. (x120).



Fig. 8. Growth of sporelings deprived of CO_2 , 48 hours. (x120).

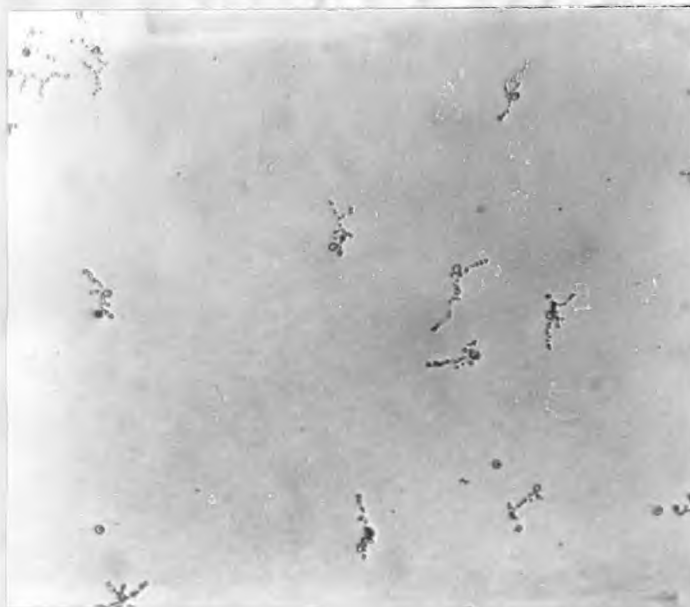


Fig. 9. Growth of sporelings deprived of CO_2 , 5 days. (x120).

However, although all viable spores may have germinated whether CO₂ was present or not, striking differences were found in the subsequent behaviour of the sporelings. The evidence for this statement is clearly shown in Figs. 3 - 9. Figs 3a and b show the growth of sporelings held in an unrestricted atmosphere. In 6 hours, those spores which had germinated had produced a visible germ tube (Fig. 3a), whose length was usually 2 - 4 times that of the parent spore; and in 12 hours these had developed the branching form shown in Fig. 3b. Later growth was rapid and within 24 - 48 hours the colony had become visible to the naked eye; the young hyphae had intertwined to give a recognisable mycelium.

Growth in a CO₂-restricted atmosphere was identical in the early stages, as shown in Fig. 4a, and the sporelings (single germ tubes) were indistinguishable from those in the control cultures. At 24 hours, some further growth had occurred, and most of the sporelings were branched; they were appreciably less well developed than the controls, but apart from this, there was no sign of abnormality. For the next 24 hours, the position remained static (Fig. 4c) and thereafter the hyphae assumed a "granular" appearance

(Figs. 4d & 9). These figures represent sporelings which have been exposed to the air at intervals (6, 24 and 48 hours) for sufficient time to allow microscopic examination. Fig. 5 shows sporelings which have been kept entirely in a CO_2 -free atmosphere for 5 - 8 days; all the same features visible in Fig. 4 re-appear here - evidently there has been germination, some growth and finally "granulation". It may be noted also that brief exposure to air (Fig. 3) did not prevent the process of disintegration - presumably the dangers mentioned above did not in fact materialise and insufficient CO_2 was taken up to have any effect.

Some attempts were made to establish the nature of the granules in the hyphae by staining with iodine in potassium iodide, cotton blue in lactophenol and Sudan III; the only conclusion reached was that at the granular stage, no protoplast was present.

One point of minor interest is shown in Fig. 6. Occasionally, a spore was found to germinate belatedly in a CO_2 -free culture and the sporeling grew quite vigorously; it may be that disintegration of the early sporelings produces (by autolysis) sufficient of some nutrient which replaces CO_2 and allows growth of the odd sporeling.

As stated above, observations concerning spore germination, as defined by the emergence of a germ tube, failed to reveal any significant effect of CO_2 on this phase of development and cultures deprived of CO_2 were frequently found to give a slightly higher percentage germination than those exposed to ordinary atmospheric conditions. One possible explanation is that the spores themselves contain sufficient reserve material to allow initiation of growth, but that in the absence of CO_2 further development is not possible; this would be quite compatible with the observations on the sporelings, but other explanations are possible. It might be that the inoculum carries with it sufficient of some "primer" to allow the germ tube to develop, and this possibility has received some consideration, as described in a later section. It could also be that the medium itself although chemically defined, prepared from purified salts, contained a sufficient amount of a "primer". The one component of the solid medium whose chemical purity cannot be vouchsafed is the agar base. In all the tests hitherto recorded, Oxoid No. 3 agar was used; and indeed this material is adequate for most purposes. It seemed worthwhile, however, to investigate the use of a more highly purified material, and Oxoid Ionagar No. 2 was

chosen. This is a product which has been purified by ion exchange, and, apart from a rather high content of Mg, is notably free from inorganic ions. Ionagar was used in the preparation of a series of media whose composition was varied by excluding one or more of the components of the usual Czapek-Dox-glucose medium, the purposes being to discover whether any component had any particularly notable role in encouraging germination, and also to see if deficiency of any component or components in the medium would give rise to abnormal growth forms (i.e. stunting and granulation) similar to those typical of CO₂ deficiency. For comparison, a similar series of tests was made with Oxoid No. 3; and the Ionagar tests were performed both in air and in a CO₂ free atmosphere. The results are summarised in Table 4 and show some interesting features. Oxoid No. 3 agar evidently contains small amounts of nutrient, for not only did spores germinate on it alone, but a visible colony, albeit very thin and spreading (starvation type) was produced; and a similar colony appeared on all the media from which glucose was omitted. Glucose alone permitted as much germination as the full medium, and although the 5 day colony remained of the starvation type, small perithecia were formed.

Experimental SectionTABLE 4

Germination of spores, and development of sporelings, on media based on Ionagar, in atmospheres (a) containing CO₂ and (b) deprived of CO₂. (Oxoid No. 3 agar included for comparison.)

Medium	% germination (16 hrs.)†	Type of Growth and Comments
Ionagar 1*+ CO ₂	26	normal colony
Ionagar 1 - CO ₂	33	granulation and stunting
Oxoid 1 + CO ₂	++++	normal colony, mature perithecia
Ionagar 2 + CO ₂	27	thin woolly colony, no granulation or stunting
Ionagar 2 - CO ₂	<1	granulation and stunting
Oxoid 2 + CO ₂	++++	normal colony few immature perithecia
Ionagar 3 + CO ₂	15	no colony, but thin mycelium: no granulation or stunting
Ionagar 3 - CO ₂	33	granulation and stunting
Oxoid 3 + CO ₃	+++	thin colony perithecia mainly initials
Ionagar 4 + CO ₂	34	very thin small colony: no stunting but some hyphae had swollen tips
Ionagar 4 - CO ₂	<1	granulation and stunting where germination occurred
Oxoid 4 + CO ₂	++++	thin colony, patchy growth

Continued.....

TABLE 4 (cont'd)

Ionagar 5, 6 + 7 + CO ₂	<<1	no colony thin individual hyphae: no granulation or stunting
Ionagar 5, 6 + 7 - CO ₂	<<1	stunting and granulation
Oxoid 5, 6 + 7 + CO ₂	++(+)	very thin colony very few peri- thecia: no stunting or granula- tion
Ionagar 8 + CO ₂	nil	-----
Ionagar 8 - CO ₂	nil	-----
Oxoid 8 + CO ₂	+	very thin colony, no stunting, perithecia or granulation

* Figures refer to composition of media, other than agar, as follows:-

1. glucose, sodium nitrate and basal salt solution.
2. glucose + sodium nitrate.
3. glucose + basal salt solution.
4. glucose.
5. sodium nitrate and basal salt solution.
6. sodium nitrate.
7. basal salt solution.
8. nil

(basal salt soln. $\equiv$ KH₂PO₄, MgSO₄·7H₂O, KCl + FeSO₄·7H₂O)

‡ Growth on the Oxoid agar plates had proceeded too far at 16 hours to permit accurate counting of germinated spores.

The greater purity of the Ionagar was reflected in the less vigorous growth of the fungus in the presence of CO_2 on all the incomplete media, compared with the corresponding cultures on Oxoid No. 3 agar media. Germination was always virtually nil on all media without glucose, and where an occasional spore did germinate, it gave rise only to long thin single hyphae. In the presence of glucose with without salts, germination was good and a small thin colony was produced; some hyphae had abnormally swollen tips, a little granulation was seen in older hyphae and no perithecia were produced. When CO_2 was excluded, germination was still more restricted, and was only significant with two media, namely the full medium and that containing glucose plus basal salts but no sodium nitrate; evidently glucose, although essential, is valueless alone in the absence of CO_2 . It may be concluded that in the presence of CO_2 glucose is essential for germination, and permits this to occur even in the absence of other constituents of the medium; but in the absence of CO_2 , glucose, though still essential, is inadequate alone. Even with the purified Ionagar and with the exclusion of CO_2 , germination occurs in the presence of glucose

and basal salts but the sporelings show the same stages as those grown in ordinary medium, resulting in granulation and death.

B. Influence of pretreatment of spores on their germination.

(Note: The experiments now to be described were intended to give a more precise idea of some of the factors which may affect germination under ordinary conditions; no attempt was made to exclude CO_2 , and in view of the negative results obtained the tests were not repeated in a CO_2 -free atmosphere.)

(i) The effect of washing the spores

Since the earliest reports of the "bios" effect, it has often been found that some stimulant which encourages growth may be carried over in inoculum to a new colony. As stated above (p.37) spores prepared by the wet method were usually washed twice with distilled water before inoculation, so that there could have been very little of the original growth medium carried over. However, some experiments were performed to see whether washing the spores did have any effect on their germination, and whether any evidence could be found to suggest the presence of a growth-stimulant transferred from the parent culture. For this purpose, a spore suspension

was made (designated unwashed); from a portion of this, the spores were centrifuged down and resuspended in the original volume of distilled water (once washed); a further portion of this suspension was centrifuged, and the spores resuspended (twice washed). The various suspensions were plated out, and germination assessed as usual by microscopic examination. The results are shown in Table 5.

In a similar experiment, spores which had been washed four times were resuspended in the supernatant fluid from the first centrifugation (i.e. that used to make up the original suspension from the slope). The percentage germination was assessed as above (Table 6).

(ii) Priming of the medium.

Pursuing their suggestion of the need for some substance arising from the spores or sporelings themselves, Darling and McArdle reported that some phases of germination and growth of sporelings of A. amstelodami are stimulated if the medium has been "primed" by a culture of the same fungus. The priming they achieved by growing colonies of A. amstelodami on Cellophane membranes laid on the surface of the medium so that diffusible substances derived from

TABLE 5

The effect of washing on the germination of spores

Time washed	% Germination	
	5 hrs.	7 hrs.
0	45	46
1	47	48
2	38	44

TABLE 6

The effect of the initial suspension fluid on the germination of spores

Time washed	% Germination	
	4 hrs.	6 hrs.
0	21	42
1	22	43
2	21	46
2*	25	47

* Suspended in the initial suspension fluid.

the growing mycelium were able to pass into the medium, while the Cellophane excluded any fungal hyphae. After 24-36 hours, the membranes were removed, leaving the primed medium ready to receive the inoculum.

In experiments of this type with C. globosum, discs of Cellophane were placed in close contact with the surface of the agar medium and a sufficient amount of standard spore suspension was run onto the surface of the Cellophane. The plates were held at 30°C for 24-36 hours, when the Cellophane discs were removed and the medium inoculated directly with a fresh spore suspension in the usual way. The results are shown in Table 7.

Growth on the primed media was, in general, more rapid, especially in the earlier stages and the total production of mycelium appreciably greater by the 13th day, when the tests were concluded. Time of appearance of immature and mature perithecia was unaffected and little difference in the intensity of sporulation was observable.

However, the validity of these results is very much in doubt, for the following reasons. Whereas Darling and McArdle had no difficulty in preventing hyphae of A. amstelodami invading the medium under

TABLE 7

The effect of "Priming" on Growth of Chaetomium globosum

Day	Colony diameter (mm.)							
	2	3	4	5	6	7	10	13
Unprimed	12.5	17	18.5	20	23	25.5	29	37
Primed	14	21	24	25	26	27	30	44

the Cellophane discs, (and an experiment confirmed this) C. globosum was found to penetrate the cellophane so that the hyphae came into contact with the agar medium. After the discs of cellophane had been removed, hyphal fragments could be observed on the medium, so that even at this stage the medium had received, as it were, an inoculum - in other words, the growth on the "primed" plates had actually been given a "start" of some 24 - 36 hours.

(iii) Exceptional grown on media in absence of CO₂

When CO₂ is rigidly excluded from the environment, growth beyond the germ-tube stage has usually been found negligible. Occasionally, however, intact perithecia would be carried over with the inoculum. On subsequent incubation in a CO₂-free atmosphere, mycelium normal in appearance was produced by the spores which germinated within the perithecia, while the single spores which germinated only produced stunted sporelings. Sporulation did not occur in those colonies, their growth was slower than that of the control colonies and much of the mycelial growth was under the agar surface.

C. Attempts to replace CO₂

Assuming that CO₂ is incorporated into some compound essential for further metabolism, it would seem an obvious step to seek for such a compound

which would allow normal development even in a CO₂-free environment. This approach has been used by earlier workers, and it may be recalled that Moss detected some positive effect of malic acid and succinic acid in permitting sporulation of Chaetomium globosum in the absence of CO₂, by his "disc" method.

Accordingly, a wide range of substances was investigated in attempts to find a CO₂-free medium in which germination would be followed by normal growth of the sporelings. Single substances, (or simple mixtures of these), were added to the medium in catalytic amounts; and since the question of permeability had to borne in mind, in some instances tests with a given substance covered a range of pH values.

The following substances, most of which have been recorded as having an influence in fungal growth, were used in these tests:-

(i) Substances of known composition

1. Carboxylic acids

Succinic acid

Malic acid

Oxaloacetic acid

Isocitric acid

2. Amino acids

Alanine

Aspartic acid

Glutamic acid

Threonine

Combinations of
these acids

Arginine

Proline

Casamino acids

3. Nitrogenous bases

Guanine

Uracil

Adenosine

Yeast nucleic acid

Alanine + adenosine

4. Miscellaneous

Fructose 1, 6 diphosphate

Riboflavin

3 Phosphoglyceric acid

Biotin

Pyridoxine

Nicotinic acid

Thiamine

p Aminobenzoic acid

Ca Pantothenate

Tween 80

All attempts to use these substances, in various concentrations and conditions as a replacement for CO₂ were unsuccessful.

(ii) Materials of unknown composition

In further attempts at replacement, more complex materials, e.g. extracts from various plant and fungal tissues, were used, and in some instances these were found capable of replacing CO₂. In these experiments, however, conclusions were usually based on the appearance of a colony visible within 48 hours, and the conditions of the spores and sporelings

during the very early period (up to 16 hours) was not investigated.

1. Yeast extract.

The basal medium, plus 0.5% glucose, was supplemented with Difco Yeast extract, 3g./100ml. of 5 control cultures, 3 had produced visible colonies within 24 hours, and the other 2 had germinated; of the 5 cultures deprived of CO₂, all had germinated and produced normal sporelings, but no visible colonies, within 24 hours. Colonies were well developed in 48 hours and growth was normal. Thus, apart from a slight lag during the very early phase, no effect resulted from deprivation of CO₂ in the presence of yeast extract.

2. Malt extract; peptone; lentil extract

The effect of these supplements was similar to that produced by yeast extract, except that early growth of the sporelings deprived of CO₂ was slower and colonies were not visible till the third day.

3. Jute extract.

Jute extract has regularly been used to supplement the Czapek-Dox-glucose medium and has a very notable effect in promoting vigorous growth and heavy sporulation of Chaetomium globosum.

A similar effect was found when spores were inoculated on to media containing jute extract and deprived of CO_2 . A colony, visible to the naked eye, was always produced within 24 hours and subsequent growth of this colony was normal.

4. Hop extract

Hop extract was prepared in the manner described (p. 36) for jute extract, and was used in a similar manner. Its effect resembled in all respects that of jute extract.

5. Staled medium

Chaetomium globosum was grown in batches of liquid medium, and after 2, 5, 8 and 10 days the mycelium was removed by filtration. To the filtrate, additional glucose, (0.5g./100 ml.) and sodium nitrate (0.2g./100ml.) together with agar (1.0g./100.) and the behaviour of spores sown on the reconstituted media was studied. No activity was observed with the 2-day and 5-day staled media, when stunting and later, granulation of the sporelings resulted. However, with the 8-day and 10-day staled media, the effect was similar to malt extract and peptone and visible colonies were produced by the third day.

6. Mycelial Extract of *Chaetomium globosum*

5-day old mycelium, grown in liquid culture was harvested by filtration and washed with distilled water before grinding in a small glass hand homogeniser. Mycelial debris was centrifuged off and the extract incorporated in basal Czapek-Dox agar.

The effect of this supplement was similar to that of malt extract, all plates producing visible colonies by the 3rd day, but subsequent growth was very poor.

7. Attempts to isolate or concentrate the active factor of jute extract

Since the jute extract was apparently able to permit germination and sporeling growth in the absence of CO₂, some attempts were made to concentrate the factor responsible. Precipitation and chromatographic methods were employed. In the latter, concentrated jute extract was run on paper chromatograms (for solvent see p. 42, Materials and Methods.) At the end of the run, the paper was dried and cut into five equal sections. Each section was extracted with distilled water, equal in volume to that of the jute extract before concentration and application to the chromatogram. This extract was then used to

suspend twice washed spores before inoculation on to basal medium. The effect of the extract was judged by comparing the germination of spores suspended in the extract with that of spores suspended in distilled water.

Fractional precipitation of the extract with ethanol was also used in some attempts to concentrate the factor. The results were quite inconclusive. On the chromatogram, the fraction with an Rf of approx. 0.6 seemed to encourage germination and a 95% ethanol soluble fraction likewise was more active than the corresponding insoluble fraction. Since a variety of spray reagents failed to indicate the identity of any component in the active fraction of the chromatogram, this line of work was abandoned.

Some of the natural extracts mentioned above have been shown to stimulate germination also under normal conditions, i.e. in an unrestricted atmosphere. The accompanying Table (Table 8) shows results obtained in a typical series of tests with these extracts.

TABLE 8

The effect of certain supplements on Percentage Germination of spores of Chaetomium globosum.

Supplement of Medium	% Germination (3 hours)
- (control)	3 - 6
Jute extract	44
lentil extract	10
yeast extract	37
casamino acids	43

D. Vegetative growth

The previous section has described experiments which showed clearly that although a proportion of the spores in an inoculum certainly germinated in the absence of CO₂, the resultant sporelings survived for only a limited time, and that an irreversible breakdown, evidenced by "granulation" of the hyphae, then took place.

The section which now follows describes some attempts to study the extent to which CO₂ affects

vegetative growth of the colonies which have passed the sporeling stage.

All attempts to study this phase of growth are handicapped by the virtual impossibility of ensuring a CO_2 -free environment, since the growing mycelium itself produces a great deal of respiratory CO_2 , which, in a closed Petri dish would tend to accumulate on the surface of a culture. One simple and obvious expedient to minimise the local concentration of CO_2 would be to invert the cultures and to grow them in vessels of sufficient size to allow a free gravitational fall of CO_2 away from the mycelium. Some tests were made in which the cultures were grown in 250 ml. conical flasks, inverted, and in some cases without the customary plug. In some, no provision was made for absorbing the respiratory CO_2 ; in others a short tube, slipped into the neck of the flask, and provided with a "fan" of filter paper (as used in Warburg respirometry), contained 10% sodium hydroxide solution. In these experiments, growth was assessed as colony diameter at intervals up to 13 days or longer; in some cases, total growth was measured as dry weight of the mycelium.

Somewhat unexpectedly, it was found that vegetative growth of the inverted colonies, when no

attempt was made to absorb CO_2 , was appreciably greater than that of colonies grown in (plugged) flasks held in an upright position. Both colony diameters and mycelial dry weights (at day 14) exceeded the controls. In the "inverted" flasks, when provision for absorbing CO_2 was made, growth was at first delayed, but by the sixth day was as vigorous as that of the other "inverted" colonies, and by the ninth day had surpassed the latter. This type of result is exemplified by the figures in Table 9.

For the growth of many microorganisms there seems to be an optimum level of CO_2 . No attempt has been made to investigate this for the vegetative growth of C. globosum, but a few qualitative tests were made in which pairs of colonies (from the same spore suspension) were grown under conditions such that one colony was always exposed to a higher concentration of the gas than the other. This was done by taking advantage of the density of CO_2 , by growing one colony in a Petri dish inverted over a deep crystallising dish of the same diameter in which the twin colony was grown. Thus the respiratory CO_2 from the upper colony would add to that of the lower, while the depth of the crystallising dish would minimise

loss of CO_2 by diffusion. No attempt was made to determine the actual concentration of CO_2 at any point in the system. Very little difference between the upper and lower colonies could be seen, but the trend was for the upper colonies always to be slightly larger (2-4mm. in diameter) than the lower. In a batch of 12 cultures, of which 50 measurements were taken during the 14 days, the upper culture was larger in 43, smaller in 4 and equal in 3.

Many experiments were performed in which spores, sporelings, or colonies of various ages were transferred from an ordinary ("unrestricted") to a CO_2 -restricted-atmosphere, or vice versa, in order to see/^{to} what degree any later growth might be affected by the change of environment.

(a) Transfer from "unrestricted" to " CO_2 -restricted" atmosphere.

In these experiments, Petri dishes containing cultures growing under normal conditions were transferred at various times to larger Petri dishes in which were containers with sodium hydroxide solution. When the transfer was made 24 hours or more after the time of inoculation, no significant difference was found in the rate or amount of mycelial growth. An example is shown in Table 10.

TABLE 9

The effect on vegetative growth of maintaining colonies in an inverted position, with and without absorption of CO₂.

250 ml. conical flask	Mean colony diameter (mm.) Days after inoculation					Dry weight of mycelium (mg.) 14
	2	4	6	8	10	
Vertical	7.5	18	22.5	30	35	17.6
Inverted, no NaOH	13	28	32	35	40	23
Inverted, + NaOH	n.v.c.	14	33	44	47	27

n.v.c. = no visible colony

TABLE 10

The effect on vegetative growth of transfer from an "unrestricted" to a "CO₂ restricted" atmosphere

Day of transfer	Dry weight of mycelium (mg.) Day 13 (mean of 3)		
		±	
2	25.3	±	0.7
3	24.6	±	1.9
4	25.3	±	0.4
n.t.	27.8	±	4.0

n.t. = no transfer

When the transfer was made less than 24 hours after inoculation, results were irregular; as would be expected, the shorter the period, the fewer and smaller the colonies produced.

(b) Transfer from "CO₂-restricted" to "unrestricted" atmosphere

20 Petri dish cultures were set up as controls in an unrestricted atmosphere, ("C"-plates) and 20 more ("D"-plates) were held from the moment of inoculation in an atmosphere deprived of CO₂. A third batch of 20 plates ("T") was prepared in the same manner as the "D" plates. At intervals up to 13 days, 2 "C" plates, taken at random, were removed, colony diameter measured and dry weight of growth determined. At the same times, 2 "D" plates were taken for microscopic observation. Of the "T" plates, 2 were taken at intervals up to 9 days and transferred to an unrestricted atmosphere (within larger Petri dishes containing no sodium hydroxide); the development of these colonies was followed for periods of up to 13 days after transfer. Tables 11a and 11b summarise the results of this experiment. (It may be remarked that none of the "D" plates gave late spontaneous development of a colony which often occurs irregularly in any batch of preparations -

TABLE 11

The effect on vegetative growth of transfer from a
 "CO₂ restricted" to an "unrestricted" atmosphere
 a) Control (c) plates and CO₂ deprived (D) plates

Days after inoculation	Control		CO ₂ deprived
	Colony diameter (mm.)	Dry wt. mg.	
2	11	0.6	35% germination
3	19	1.6	sporelings
4	22	3.2	granulation
6	24	3.8	"
9	26	8.5	"
11	27	14.1	"
13	34	19.8	"

b) Transferred (T) plates

Day of transfer after inoculation	Colony Diameter after transfer				Dry wt. (mg.)
	Day				
	2	6	9	13	Day 13
1	17	26	30	36	14
2	18	24	27	35	21
3	10	25	31	37	23
4	(growth)	24	31	34	18
5	12	23	29	37	22
6	12	24	30	36	20

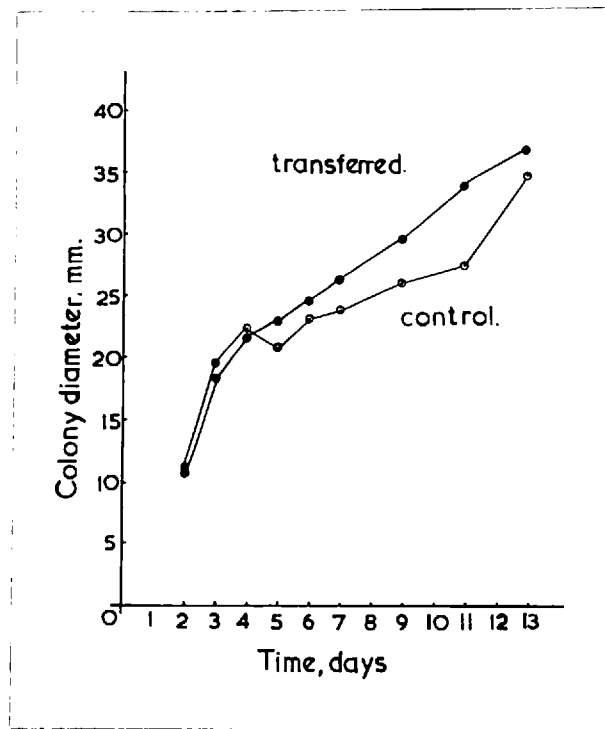


Fig. 10. Graph of mean colony diameter of control and transferred colonies against time.

see p. 59). It appeared that, provided sufficient spores remained viable after being held in a CO_2 -free atmosphere, vegetative growth, once begun, was quite normal in amount and rapidity (see Fig. 10).

c. Formation of perithecia

Moss (44) studied the effect of CO_2 and of some possible substituents for the gas on perithecial production; the object of his method was to dissociate vegetative growth from sporulation, and this he attempted to do by taking portions of mycelia which had not yet produced perithecia from parts of the culture where the supply of nutrient (glucose) was almost exhausted. This implied that further development, e.g. of perithecia, depended upon mobilisation of the internal reserved (? glycogen) of the mycelium. Since removal of CO_2 from the environment suppressed sporulation, it would seem that, either this mobilisation, or the subsequent utilisation of the metabolites produced, was still CO_2 dependent. This fact allowed Moss to investigate the replacement of CO_2 by other possible intermediate metabolites.

In this work, several scattered observations suggested that in a normally growing colony, the level of CO_2 in the environment had some effect on perithecial development, and indeed in some instances

temporary deprivation of CO_2 at certain stages of growth still influenced the subsequent growth of perithecia even though vegetative growth seemed to have recovered from the check caused by lack of CO_2 and to have become normal once more. If this is so, many interesting problems arise. It was not possible to investigate this matter in detail, and only a brief resume will be given here of the observations made in the course of what must be regarded as an exploratory investigation.

(a) Effect of transfer of a colony to a CO_2 restricted atmosphere at different stages of growth

The results summarised in Table 12 refer to the experiments described on pages 78 which record the effect of a restricted supply of CO_2 on vegetative growth. Here, some effects on sporulation are noted.

(b) The results recorded in Table 13 were derived from the experiments recorded on pages 79 in which colonies on Czapek-Dox glucose agar were grown in one or both parts of a container consisting of a Petri dish inverted over a crystallising dish.

(c) Reference has been made to the fact that on media enriched by various extracts of natural materials, and particularly of jute, growth seemed to be as

TABLE 12

The effect of transfer of a colony to a "CO₂ restricted" atmosphere at different stages of growth

a)

Time of transfer (Hours after inoculation)	Vegetative growth	Perithecial Growth
0	nil	nil
3	weak	initials only
24	vigorous	initials and immature
48	vigorous	immature
control (not transferred)	vigorous	mature

b)

Time of transfer (Days after inoculation)	Dry weight (mg.) of perithecia	and type (Day 13)
3	1.8	Immature
5	3.0	Mature
7	3.5	Mature
control (not transferred)	3.4	Mature

TABLE 13

Average dry weight of perithecia on day 13.

Colony in upper dish only	2.6 mg.
Colony in lower dish only	3.5 mg.
Colony in both dishes a) upper dish	2.8 mg.
b) lower dish	nil

vigorous in CO₂ restricted atmospheres as in unrestricted ones. As to perithecial production, a much more striking effect was found when the media contained jute extract; the total weight of fungal tissue produced in 13-14 days was similar whether or not CO₂ was restricted by the presence of an absorbent, but weight of perithecia produced was very much greater when an absorbent was present. In 4 colonies thus grown, in an unrestricted atmosphere the average total dry weight per colony was 26.9 mg., of which perithecia accounted for 8.2 mg., while in the presence of NaOH solution throughout the growth period, the corresponding figures (6 colonies) were 25.8 mg. and 13.1 mg.. This effect was not found when the basal medium was augmented with biotin, nor with a mixture of biotin, thiamin, riboflavin, nicotinic acid, pyridoxine, calcium pantothenate, p-aminobenzoic acid and inositol. Enrichment with yeast extract produced indefinite results.

PART IIFIXATION OF CARBON DIOXIDE BY CHAETOMIUM GLOBOSUM:EXPERIMENTS USING ^{14}C AS TRACER

Moss (44) reported a preliminary experiment using gaseous $^{14}\text{CO}_2$, in which he showed that C. globosum was capable of fixing CO_2 , and that the form into which it is fixed is, in part at least, respirable.

In view of the effect of CO_2 on germination and growth reported above, it seemed highly desirable to extend the work on CO_2 fixation using ^{14}C as tracer, and to elucidate, if possible, the pattern of fixation.

Although the use of ^{14}C as a tracer is now a very common technique, certain difficulties presented themselves when its application to the present problem was undertaken. Ideally one would wish to identify the initial reaction by which CO_2 is incorporated into the tissue. All the evidence would suggest that this is an extremely rapid reaction (cf. studies on photosynthesis in algae) and that the initially formed compound is immediately utilised in a further reaction. Evidently, the closer one could approach to the sporeling phase, and even the earliest moments of this phase, the less likelihood there would be of labelled products of secondary origin obscuring the product of the primary reaction. Equally evident is

the fact that this ideal is a practical impossibility, if only on account of the very large number of sporlings that would be necessary to give the quantity of material required for analysis.

In order to gain experience of the fractionation and chromatographic techniques colonies three to five ^{old} days/which had been exposed to $^{14}\text{C}\text{O}_2$, for a suitable period were used in the first experiments. Moss (44) employed a method whereby gaseous CO_2 was generated within a closed culture vessel, but in the present work the ^{14}C was supplied in the form of $\text{NaH}^{14}\text{CO}_3$, a solution of which was injected into the medium at an appropriate time. This procedure formed the basis of experiments whose objective was to identify as far as possible all the compounds into which the carbon from CO_2 is incorporated. A culture in 20 ml. liquid Czapek-Dox medium in a 150 ml. conical flask was incubated for two days, the flask being closed with a cotton wool plug. Th plug was then replaced with a "Suba Seal" rubber stopped and the flask flushed out with CO_2 -free air for 5 minutes. 1.0 ml. $\text{NaH}^{14}\text{CO}_3$ solution (400 $\mu\text{g}.$; 10 μC) was injected into the culture by means of a sterile syringe and the culture incubated for a further 3 days. Three control flasks were set

up under identical conditions, except that $\text{NaH}^{12}\text{CO}_3$ solution was used.

After incubation, the radioactive mycelium was harvested by centrifugation and analysed as described above (Materials and Methods Section, p. 39). The dry weight of mycelium produced was obtained from the mean of the dry weights of mycelium from the control flasks. The results from a typical experiment are shown in Table 14.

A similar experiment was performed in which the NaHCO_3 solution was added to the culture flask immediately after inoculation, and the mycelium harvested after 3 days incubation. The results are shown in Table 14.

Comparison of these results shows some interesting features. First of all, the total radioactivity recovered was greater from the 0-3 day fixation period than from the 2 - 5 day period, even though the dry weights are comparable. This is similar to the finding of Barinova (43), who showed that for Aspergillus niger there is no proportionality between CO_2 uptake and growth.

Examination of the distribution of radioactivity in the fractions obtained from the mycelium

TABLE 14Fixation of CO₂ by C. globosum : 0 - 3 days + 2 - 5 days

Fraction	0.3 days		2 - 5 days			
	c.p. 100 secs	%	c.p. mg.	c.p. 100 secs	%	c.p. mg.
Cold TCA				990	8.3	75
25% Ethanol	1371	7.0	118			
75% Ethanol	1670	8.5	122	750	5.2	57
Ethanol/ Ether	135	0.7	12	40	0.3	3
Hot T.C.A.	5323	27.2	455	6240	40.5	473
Protein Residue	11092	56.6	948	7050	45.7	526
Total	19591			1509.0		
Dry wt. mg.	11.7			13.2		

TABLE 15Fixation of CO₂ by C. globosum; Jute extract added to the medium

Fraction	0 - 3 days		
	c.p. 100 secs.	% cp.	mg. dry wt.
25% Ethanol	1613	4.0	63
75% Ethanol	2740	6.9	107
Ethanol/Ether	435	1.1	17
Hot TCA	11800	29.8	461
Protein fraction	23040	58.2	900
Total	39628		
dry weight (mg.)	25.6		

in the two experiments showed the same result, except for the hot T.C.A. fraction, where the radioactivity was higher for the 2-5 day period than for the 0-3 day period. On a counts/mg. dry weight basis, however, the values for the two periods were almost identical.

Analysis of the fractions.

1. 25% Ethanol, 75% Ethanol and Ethanol/Ether Fractions.

No attempt was made to determine the location of the radioactive label in the first three fractions due to the low level of radioactivity.

2. Hot T.C.A. Fraction.

After ether extraction and hydrolysis with 0.1N HCl at 100°C for 30 minutes, the hydrolysate was concentrated in vacuo and analysed by ascending chromatography in butanol/ 0.8N HCl. Autoradiographs prepared from these chromatograms showed that the radioactivity was present in four different compounds, viz. adenine, guanine, cytidylic acid and uridylic acid. The identity of these compounds was confirmed by comparison of their Rf. values with those of known standard substances chromatographed under identical conditions. The amount of radioactivity in adenine and guanine was approximately equal, as judged from the

radioautographs, and was greater than that found in cytidylic and uridylic acids, although the radioactivity in the last two compounds was also approximately equal.

3. Protein Residue.

The residue was hydrolysed with 6N HCl in a sealed tube for 4 hours at 108°C and the residual HCl removed by evaporation to dryness three times in vacuo. Two dimensional chromatograms of the hydrolysate were made in butanol/formic acid/water and phenol/ammonia/water. Radioautographs prepared from these showed that the following compounds had taken up radioactivity, the relative amounts of which (indicated by + signs) were estimated visually from the density of the spots on the radioautograph.

Aspartic acid	++++	Isoleucine	++
Glutamic acid	++++	Alanine	trace
Arginine	+++++	Glycine	trace
Threonine	+++	Methionine	trace
Proline	++	Lysine	+

There were two other spots which showed slight traces of radioactivity. One corresponded in chromatographic behaviour to α -aminobutyric acid, while the other, which did not react with ninhydrin,

had the following Rf. values:

phenol/ammonia/water	0.95
butanol/formic acid/water	0.90.

Since jute extract, incorporated in the medium, permits vigorous growth of C. globosum even in the presence of a CO₂ absorbing material, a long term fixation experiment was carried out in which the medium was enriched with jute extract at the usual concentration. The bicarbonate solution was added immediately after inoculation and growth was allowed to proceed for three days as before, when the mycelium was harvested and analysed. Results are shown in Table 15. It will be seen that just over twice as much radioactivity was recovered from the mycelium which had been incubated in the presence of jute extract than from that grown on the basal medium. However, growth, assessed on a dry weight basis, was also increased by the same amount, and when the results are expressed on a counts per mg. dry weight basis, it can be seen that the presence of jute extract did in fact slightly depress the apparent fixation of CO₂. On analysis of the mycelium, this depression was found to be in the 25% ethanol and protein fractions

only, the other three fractions being almost identical with the control fractions.

No difference in composition was observed between the hydrolysates of the hot T.C.A. and protein fractions of the control and jute extract mycelia after chromatography and preparation of radioautographs of the hydrolysates.

To follow up previous observations on CO_2 fixation, some short term (2 hour) fixation experiments have been carried out. The results reported here are of preliminary experiments in this direction; work is still continuing, and there is scope here for further more detailed experiments.

For these experiments, a vegetative inoculum (see p.37) was used. 5ml. of this inoculum were added to 5ml. double strength Czapek-Dox medium in a 50ml. conical flask. The culture was preincubated with gentle shaking on a water bath at 30°C for thirty minutes. The flasks were then removed from the water bath, the $\text{NaH}^{14}\text{CO}_3$ solution added and the flask sealed with Parafilm. Incubation was then continued with gentle shaking on the water bath for two hours, after which the flasks were briefly flushed out with air before the mycelium was separated from the medium by

centrifugation. The dry weight of the mycelium used was calculated from the weight of aliquots of the inoculum, increase in dry weight during the two hour incubation period being insignificant.

The first experiment was designed to find the optimum pH value of the medium for CO₂ fixation. Double strength medium was adjusted to four different pH values and inoculated and incubated as described above. The change in pH of the medium immediately after inoculation was noted. 0.1ml. NaH¹⁴CO₃ solution (2μC) was added to each flask. (No pH change here). The mycelium was not fractionated as in the long term experiments, but was hydrolysed directly in 6N HCl in a sealed tube at 108°C before removing excess HCl by evaporating to dryness three times in vacuo. The final residue was taken up in 1.0ml. 50% ethanol prior to assay of radioactivity. The final pHs of the media were not measured, but from the results it can be seen that maximum CO₂ fixation under these conditions occurred when the initial pH of the medium was 5.7, as shown in Table 16. Accordingly, this value was adopted for the initial pH of the medium in all subsequent experiments.

As was pointed out in the introduction :

TABLE 16The effect of pH on short term CO₂ fixation

Initial pH of the medium	pH after addition of the inoculum	Total radioactivity c.p. 100 secs.
4.5	5.0	2568
5.7	5.9	2650
6.5	6.7	2175
7.8	7.6	1860

TABLE 17The effect of various additions to the medium on CO₂ fixation

ADDITION	Total radioactivity recovered c.p. 100 secs.	% depression of fixation
nil (control)	5260	-
aspartic acid	1530	70.9
oxaloacetic acid	4510	14.3
adenine + uracil	4360	17.1

a valuable way of ascertaining or assessing the role of any compound in CO_2 fixation is to incorporate the substance in the medium employed during the fixation experiment, when comparison of the amount of CO_2 fixed in the presence and absence of the compound can be made.

This has been carried out with C. globosum so far with three compounds, namely aspartic acid, oxaloacetic acid and a mixture of adenine and uracil.* Oxaloacetic acid and aspartic acid were incorporated into the medium at a level of 2mg./ml. and adenine and guanine at 0.5mg./ml. each. 0.2ml. $\text{NaH}^{14}\text{CO}_3$ solution (4 μC) were added to each flask after preincubation for thirty minutes, and fixation was allowed to continue for two hours, when the mycelium was harvested by centrifugation, washed with 0.9% saline solution and hydrolysed complete as before. The results are shown in Table 17.

* In the same experiment, arginine, the compound into which most radioactivity is incorporated in the 3 day fixation period, and jute extract were incorporated into the medium, but the results were lost due to tubes breaking in the centrifuge.

DISCUSSION

The results reported in the first, mainly descriptive, parts of the preceding section show quite plainly that CO_2 (or bicarbonate ion, under the experimental conditions regarding pH) is essential for all, or almost all stages of growth of Chaetomium globosum. A possible exception may be the very earliest stage of germination, for even when the most stringent precautions were taken to exclude CO_2 from the environment a considerable proportion of the spores in any given inoculum produced apparently normal germ tubes, from which sporelings quickly developed. However, in very rare instances only did these sporelings survive for more than 48-60 hours under CO_2 -restricted conditions. (This term is used in preference to " CO_2 -free" since it is obviously impossible to ensure the latter state all the time and in all locations.)

Several interesting features are associated with the production of the germ tube and the sporeling. Firstly, on many occasions it was possible to hold an inoculated plate (Czapek-Dox-glucose-agar culture) in a closed container in the presence of NaOH, for as long as 10-14 days without any visible mycelium appearing. Yet at least some of the spores retained their

viability, for after removing the NaOH, colonies rapidly formed and grew in a normal manner. Although such plates failed to produce visible colonies so long as CO₂ was excluded, microscopic examination of apparently non-germinated inocula has always revealed that some of the spores have in fact reached the sporeling phase. Once granulation has occurred, however, the sporelings cannot recover, and it follows therefore that any colonies which appear when air containing CO₂ is admitted after more than four days or so must have developed from spore which for some reason remained quite dormant during the period when CO₂ was excluded (There is a possibility that when the sporelings "granulate" and finally disintegrate, they could liberate into the medium some product of autolysis which would replace CO₂, and encourage germination of neighbouring dormant spores. No indication of such an effect has been seen.)

This seeming irregularity in behaviour of spores calls for some explanation, even apart from any effect CO₂ may have on germination, and various possibilities suggest themselves. It is conceivable that, in a suspension containing a large number of spores, individual spores may possess varying

capacities for germination at a given moment; or in other words may have reached different stages of maturity (whatever that may imply in terms of biochemical mechanisms), which will influence their ability to respond to conditions which permit germination. When a suspension is prepared from a mature culture, it is not possible to select perithecia all of the same degree of maturity, and for this reason alone it seems likely that no suspension will be strictly uniform in respect of germinative capacity*.

* It may be pointed out also that the rate of germination is affected by the age of the slope from which the spore suspension is prepared. Figures quoted below in Table 18 are of percentage germinations (at varying times) of spores derived from slopes of different ages.

Table 18

age of slope (days)	% germination of spores		
	3 hrs.	4 hrs.	6 hrs.
9	6.6	12.0	19.8
16	17.1	22.9	35.8
23	22.9	28.2	46.6
33	28.7	43.3	56.9

In short, when the spores of an inoculum are deprived of CO_2 , some of them will be able to develop and produce one or two small hyphae; these rapidly granulate and disintegrate. The remainder of the spores will fail to germinate at all, and remain dormant while still retaining their viability; on exposure to an unrestricted atmosphere, some at least of these may respond and give a normal colony. It might be suggested that spores of varying degrees of maturity need different levels of CO_2 even for the initial phases of germination. If this be so, the slightly higher percentage germination noticed when CO_2 was restricted could be due to the fact that spores of a certain maturity may even be stimulated by low CO_2 concentrations.

This idea of a connection between the degree of maturity of the spore, its capacity for germination, and its response to CO_2 would be very difficult to uphold experimentally. But there are other possibilities which may be considered and tested, and the first of these is whether an inoculum can carry with it from the parent culture some stimulant which may encourage germination even to the extent of lessening the need for CO_2 . Investigations into the effect

of repeatedly washing the spores before preparing the inoculum indicated that such stimulation is unlikely; this is most strongly indicated by the fact that spores, washed twice but then resuspended in the original liquid, behaved similarly to those also washed twice but resuspended in distilled water. This is contrary to the classic finding of Wildiers (96; 1901) who was able to attribute the favourable effect of a heavy inoculum (yeast) to its carry-over of micronutrients.

Another possibility is that the spores themselves give rise to an essential growth factor (or sporeling factor) which is required in a critical concentration. This could be a substance, pre-formed within the spores, which, when the spores are exposed to suitable conditions regarding, e.g. moisture, diffuses through the spore walls and in so doing modifies the wall and facilitates the emergence of the germ tube. Or it could be that a product of the early metabolism of the spores, but necessary for their continued development is concerned. This latter suggestion was made by Darling and McArdle (95) to explain their observations on the development of sporelings of Aspergillus amstelodami, and they pointed out that the simplest compound that would fulfil the

requirements of their "sporeling growth factor" was CO_2 . Whatever the source of the supposed stimulant, it seems reasonable to suppose that the greater its concentration, or the sooner a critical concentration is reached, the greater will be the effect on germination and on sporeling growth.

The use of a denser inoculum would be conducive to the more rapid attainment of a critical concentration as would also the use of a smaller volume of culture medium (smaller Petri dish), whereby diffusion of the factor would be restricted. Some experiments were performed in which measured amounts of a standard inoculum were spread over the surface of media, of uniform depth, contained in Petri dishes of different diameters. Indications were obtained that germination took place several hours earlier in $1\frac{1}{2}$ " dishes compared with $3\frac{1}{2}$ ", but these observations were not followed further. However, this suggested concentration-effect might well explain the fact, noticed repeatedly, that on the occasions when growth beyond the sporeling phase took place in absence of CO_2 , it was associated with the presence of unbroken perithecia or of local aggregations of spores. The suggestion is therefore made that respiratory CO_2 is produced at a very early stage, and

that when many spores lie close together, as would be the case when an intact perithecium broke open, a local concentration of CO_2 develops, sufficient to permit the normal growth of at least a proportion of the sporelings. A point will be reached where sufficient CO_2 is being produced to allow full development of the colony, since it seems impossible in practise to remove CO_2 to a degree which will stop the growth of an already developing colony (p. 86). CO_2 , produced by the respiration of the spores - which it is reasonable to assume will commence as soon, or almost as soon as any energy requiring processes are initiated within the spore - seems to fit all the experimental observations on the effects of dilution of the inoculum.

It seems established that germination in the true sense, if it calls for the presence of CO_2 at all, can certainly take place under a CO_2 -tension much less than that of the atmosphere; and in fact a diminished tension may be preferable to an atmospheric one. It would not be possible to say that complete absence of CO_2 or bicarbonate permits germination, since this state is most probably unattainable in practice. However, if such were the case, it would indicate that the spores themselves contain a

reserve or potential reserve of some material which is able to replace CO_2 during the early stages of germination.

Certain observations, now to be commented upon, make it unlikely that any appreciable amounts of such a reserve are present in the spores. In the first place, no sign of germination whatsoever is observed when water Ionagar (with no additions) forms the medium; this is the case whether or not CO_2 is available. Distilled water is similarly ineffective in allowing germination. Further, when Ionagar is used, and CO_2 is excluded, there is virtually no germination (i.e. less than 1%) in the presence of glucose or of glucose and sodium nitrate. These instances of failure to germinate could be due either to lack of any CO_2 -replacing reserve as suggested above, or to lack of any reserve of energy (or energy yielding metabolite) in the spores. There could indeed be a connection between the two, since utilisation of carbohydrate or fat as a prime source of energy would be expected to result in the simultaneous liberation of CO_2 .

Lacking any internal energy-producing (or CO_2 -producing) reserve, the source of these essentials must be sought among the constituents of the medium.

Glucose suggests itself as an obvious possibility, and the idea is favoured by the fact that on an Oxoid No. 3 agar medium it seems to play the predominant role in stimulating germination; so long as it is present, germination reaches the same level as with the full medium - other components can be omitted without diminishing the percentage germination, but omission of glucose results in a substantially lower percentage germination. Similarly on Ionagar, germination equal to that on the full medium occurs when the agar contains only glucose or glucose plus sodium nitrate; omission of glucose reduces germination to virtually nil. But this effect of glucose is only observed in the presence of CO_2 ; in the absence of the gas, neither glucose alone nor glucose plus sodium nitrate permit germination. If therefore glucose is essential as a source of energy, this energy can be mobilised only in the presence of CO_2 . And it seems that Oxoid No. 3 agar contains some unknown component which renders it much more effective in permitting (or stimulating) germination than Ionagar; even in the most favourable conditions (i.e. in the full medium) the percentage germination is appreciably greater on Oxoid agar - 50-60% compared with approx. 35% in otherwise similar circumstances. A

notable difference in the composition of the two agars is that Ionagar contains much less nitrogen, so possibly Oxoid No. 3 agar contains some amino acid or other nitrogen compound which is responsible for the greater activity.

It is also possible that Oxoid agar resembles certain other products of natural origin in containing some undefined substance which encourages growth of microorganisms. Experiments now recorded have shown that extracts of hops, yeast etc., and above all of jute, do possess this property. Jute extract has long been known to encourage growth and fruiting of C. globosum, and has in fact been regularly added to the Czapek-Dox medium in which vigorous cultures have been maintained. Its capacity for stimulating fruiting can be accounted for by the presence of hexose phosphates, supplemented by Ca ions; but experiments have failed to reveal any connection between hexose phosphates and spore germination. While it may be that the activity of jute extract results from the presence of a particular component of the extract, it is also possible that its influence on spore germination is indirect, and is associated with the production of a suitable concentration of respiratory CO₂

in the environment of the spores or sporelings. It undoubtedly stimulates vegetative growth very markedly and in so doing must stimulate respiration; hence the presence of additional CO_2 . This may reach a sufficient level in the immediate neighbourhood of the 24-48 hour sporelings (because of the physical impossibility of complete and instantaneous removal) to tide them over the critical period of growth, when they seem most sensitive to a lack of the gas - the moment, in fact, when granulation occurs unless CO_2 is present. This interpretation is perhaps supported by the fact that so far as post-sporeling vegetative growth is concerned, absorption of CO_2 is actually favourable to increased growth (p. 80). - a fact which could be explained on the grounds that excess CO_2 is inhibitory to growth, and that in the presence of absorbent the CO_2 concentration is kept within the optimum range. There is thus no need to postulate any special germination-stimulating factor in jute or other natural products; the effect of the extracts could be due solely to their part in maintaining, more or less by chance a suitable level of CO_2 in the environment of the growing colony.

It is of interest to compare the present observations with those of Darling and McArdle. These

workers explained the abnormal growth under certain conditions of the mutant strain of Aspergillus amstelodami as being due to two factors. The first was considered to be some genetic defect in a mechanism responsible for the supply of energy and of basic materials necessary for synthesis (growth); the second factor was a lack of CO_2 , considered to be essential for the production of normal branching of the growing mycelium. As regards the first, the defect could be overcome by ensuring that the medium was "primed" with a sufficient amount of some low molecular weight compound produced and excreted into the surrounding medium by a vigorously growing colony. The deficiency was not absolute, for even in the absence of the primer growth did take place, but consisted only of a single elongated hyphae, almost entirely unbranched. Further, even a single spore was found capable of rapid (and apparently normal) growth if it was seeded on a tiny block (2mm. cube) of agar held in a moist chamber - that is, in the limited volume of medium, diffusion was also limited and a sufficient concentration of primer accumulated.

An increased supply of CO_2 in the atmosphere (up to 10% v/v) caused an immediate, dramatic branching

of the single unbranched hyphae. But branching, although delayed, also occurred in time in the "single hyphae" colonies, since a time came when these were sufficiently vigorous to produce a level of respiratory CO_2 high enough to cause branching: these colonies "went on to sporulate and eventually caught up with the others" (i.e. the normal colonies).

Comparing these results with the present ones, similarities and differences may be observed. There is a similar retardation of growth when a light inoculum is used, or when the concentration of spores is low relative to the volume of medium. There is the possibility of encouraging growth from a light inoculum by priming the medium before inoculation. There is the need for CO_2 either for growth as such or for later branching of the young hyphae. In regard to the latter, both organisms normally reach a condition where enough CO_2 is being liberated by respiration of the organism to fulfil all the requirements for growth and/or branching.

In the matter of granulation of sporelings deprived of CO_2 , it may be pointed out that Darling and McArdle did not investigate the effect of depriving their cultures as completely as possible of the gas.

The conditions giving rise to granulation were, therefore, never fulfilled, and it may be that if CO₂ were rigidly excluded, A. amstelodami also would show this effect. One might suggest very tentatively that although Darling and McArdle distinguish between the "growth" factor and the "branching" factor, and identify the latter with CO₂, this same compound in a lower concentration is, in fact, the growth factor also. An isolated elongating hyphae might produce enough respiratory CO₂ to allow slow, starvation growth to proceed, but not enough to promote any significant branching. These authors mention 10% CO₂ as being effective for causing branching, and this is a very high concentration: they do not record any tests using a lower concentration, but even if 1% was effective, it would be a very high level to be reached in the neighbourhood of a single sporeling or hypha.

All attempts to replace CO₂ by possible intermediates failed so far as sporelings were concerned. This is unlike the experience of Moss (44) who found that succinic acid (and to a less extent malic acid) was partly able to replace CO₂ for production of perithecia. Atkinson (private communication) has informed the writer of a report that N-methyl

nicotinic acid (trigonellin) stimulates branching in Pellicularia filamentosa; so far, it has not been possible to obtain a sample of Trigonellin for testing.

Little comment is called for concerning the experiments with vigorous vegetative mycelia. It seemed that if CO_2 was essential for continued growth, more than enough was always available as a result of respiration, for no attempt to halt growth by restricting the level of CO_2 was successful. The fact that labelled CO_2 was taken up by growing colonies, and by homogenates of the mycelium, strongly indicated that although it was not possible in practice to halt growth by removing CO_2 , the gas still remains essential for vegetative growth as for sporeling development and for sporulation.

Nevertheless, there are indications that it is possible to reach a level above which any increase in CO_2 concentration is inhibitory to growth.

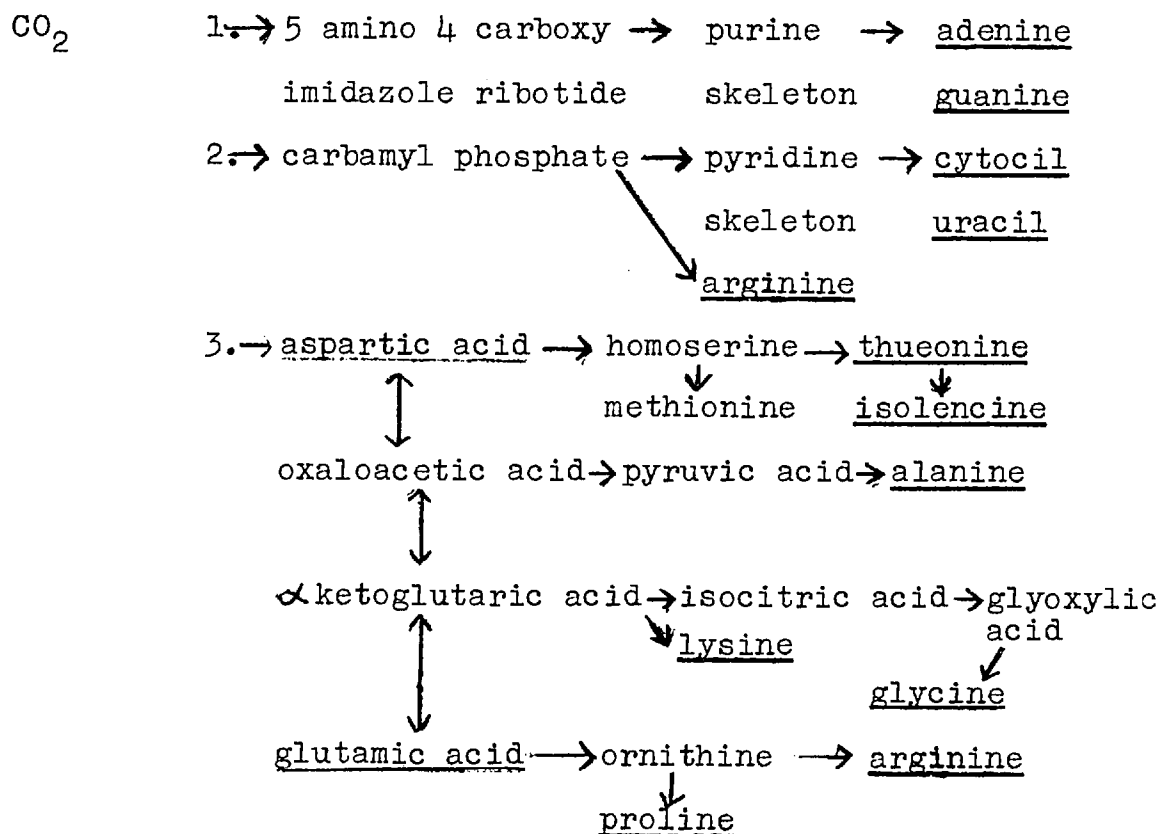
As regards sporulation, here again enough CO_2 is produced in a normally growing colony to supply all requirements, and it is only under rather exceptional conditions such as those employed by Moss that sporulation can be prevented by restricting CO_2 . It appears that the requirement of CO_2 for sporulation can

Experiments with labelled CO_2 have shown that C. globosum is capable of fixing CO_2 in a manner comparable with other heterotrophic microorganisms, as reviewed in the introduction to this thesis.

A disadvantage of the experimental method here used is that in order to obtain enough material for convenient handling (fractionation etc.), it is necessary to expose the growing mycelium to $^{14}\text{CO}_2$ for comparatively long time (72 hrs.), and during this time it is almost certain that a considerable amount of randomization of the ^{14}C will occur through the operation of the various cycles of metabolism. Experience in the field of photosynthesis has shown that, with *Chlorella* for instance, a few seconds only will suffice for the passage of carbon from $^{14}\text{CO}_2$ into a number of sugars and aminoacids, although only a single receptor is concerned. While it may be that the fixation and transfer systems of fungi are less active than those of photosynthetic organisms, it would obviously be necessary to devise experiments based on minutes or even seconds rather than on hours. This could be done only by working with very large quantities of spores and sporelings, and in the present circumstances it was reluctantly decided that any attempt to do so would present too many difficulties. The

experiments with homogenates met the difficulty only to a limited extent. It is true that the time of exposure was greatly reduced (2 hrs.) but even so was sufficient to allow as wide a distribution of the ^{14}C as that found in the experiments with growing mycelium. Neither procedure gave any information concerning the primary mechanism of fixation, and it is just this which would be of such great interest.

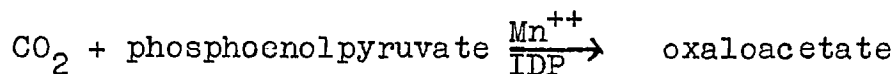
The results obtained in the present experiments are consistent with the following known biosynthetic pathways, shown below in outline:-



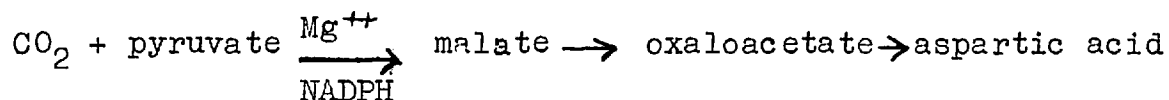
The heavy labelling of arginine can be explained by the fact that it receives a "double dose" of radioactivity; the ornithine part of the molecule having radioactivity derived from glutamic acid and the carbon atom of the amidine moiety being derived directly from CO_2 by fixation into carbamyl phosphate.

The aspartic acid plays a central part in CO_2 fixation is demonstrated by the depression of fixation (about 70%) during the two hour fixation period when it is incorporated in the medium.

The surprising feature of this experiment is the small amount (about 13%) of depression of fixation in the presence of oxaloacetic acid, a member of the Krebs cycle intimately related to aspartic acid, into which it is converted by transamination, and the reported first product of the fixation reaction:



It is also involved in the conversion of malic acid produced by the malic enzyme CO_2 fixation reaction to aspartic acid:



Two possibilities suggest themselves as explanations for this apparent anomaly. The first is

that a permeability factor is involved which prevents utilisation of oxaloacetate by C. globosum; but this seems unlikely since Moss (44) and Chatterji (97; 1961) have both shown that the fungus possesses the ability to utilise a wide range of di- and tri-carboxylic acids associated with the Krebs cycle; they did not, however, use oxalacetate.

The second is that the first free product of the reaction is indeed aspartic acid, and if oxaloacetate is involved as intermediate it is in a closely bound form and not released in free form. In the absence of further experimental evidence, this must remain speculative.

The fact that no label appears in serine and only a trace in glycine can be taken as an indication that the ribulose diphosphate carboxylase fixation system is not present in this fungus, since this reaction gives rise to 3-phosphoglyceric acid, a direct precursor of serine and hence of glycine. The trace labelling of glycine and alanine can be explained in the above scheme.

The results obtained in the fixation experiment in the presence of jute extract are very significant. The increase in CO₂ fixation observed in the

presence of jute extract is only indirectly attributable to the extract; the extract greatly increases vegetative growth and hence, as a result increases CO_2 fixation. This is witnessed by the fact that on a "radioactivity per mg. dry weight" basis are very comparable and no difference was observed in fixation products.

These observations lend weight to the hypothesis proposed earlier (p.110) that jute extract does not supply a substance necessary to replace CO_2 , but instead contains substances capable of increasing vegetative growth, and hence respiratory rate and level of respiratory CO_2 available to the fungus.

Repeated attempts have been made throughout the course of this work to find a substance or combination of substances which will, when incorporated into the medium, allow normal growth of the fungus in the absence of CO_2 . All attempts to replace CO_2 with chemically defined compounds have met with failure, even though some of these compounds have been reported by various authors to replace the CO_2 requirement of other microorganisms, or are CO_2 fixation products of this and other organisms, and success has been obtained only with a few natural extracts.

However, as has been mentioned previously in this discussion, this effect may not be due to a "replacement" effect but instead to an "increased supply" effect.

The basis of all these replacement experiments has been the assumption that CO_2 is necessary for the fungus as an essential in some biosynthetic pathway. The product of which reaction is also essential for the fungus. Since none of the identified products of CO_2 fixation has been shown to be able to replace CO_2 , although an unidentified fixation product may be responsible for the effect, the possibility exists that the requirement for CO_2 is of a more fundamental nature.

Recently, observations have been published on the effect of CO_2 on mitochondria and mitochondrial adenosine triphosphatase. Löw, Krueger and Zeigler (98 ; 1961) reported that the efficiency of phosphorylation of submitochondrial particles from beef heart is improved by the presence of small amounts of CO_2 because of a decrease in the rate of oxidation. Racker (99 ; 1962) reported that HCO_3^- stimulates the ATPase activity of a protein preparation from beef heart mitochondria, and Fanestil, Hastings and Mahwold

(100; 1963), in a comprehensive study of the effect of varying environmental CO_2 concentrations on the ATPase activity of rat liver mitochondria, found that within the physiological range of CO_2 concentration and pH the ATPase activity is increased with increasing CO_2 concentration. They state "it is tempting to speculate that the ubiquitous substance, CO_2 , plays a significant role in the control of mitochondrial oxidative phosphorylation".

If therefore, this speculation should be correct, it would form the basis of an explanation of the observed effects of CO_2 concentration whereby both to small and to large amounts of CO_2 upset the oxidative phosphorylation control mechanism of mitochondria and thus the energy production of the cell. This suggestion must, however, in the absence of further experimental evidence, remain speculative, but it does provide another viewpoint on a very interesting and complex problem.

SUMMARY

1. A review of the literature appurtenant to the effect of CO_2 on germination, growth and sporulation of, and to the fixation of CO_2 by, microorganisms has been presented.
2. The rate of germination of spores of Chaetomium globosum was not adversely affected by removal of CO_2 from their environment; in fact a slight stimulation was observed. It was shown that this could not be due to carry over of nutrients from the parent culture in the spore suspension fluid.
3. CO_2 was, however, found to be essential for further growth of the germinated spores past the sporeling stage. In the absence of CO_2 , the sporelings developed a "granular" appearance by the fourth day, no protoplast was demonstrable and they failed to grow when transferred to normal atmospheric environment.
4. The typical type of "stunted" growth resulting from the removal of CO_2 from the culture could not be reproduced by omission from the medium of one or more of the other components of the medium in the presence of CO_2 .
5. All attempts to replace CO_2 by defined chemical compounds were unsuccessful. The only

substances which would permit normal colony growth in the presence of a CO₂-absorbent were natural extracts, including jute, hop, yeast and malt extracts.

6. Using labelled CO₂, it was possible to demonstrate fixation of carbon dioxide by Chaetomium globosum. Fixation of CO₂ into purines, pyrimidines and amino acids was shown to occur.

7. The relationship between carbon dioxide and growth and metabolism of Chaetomium globosum is discussed, and an attempt has been made to assess the results in the light of current knowledge.

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ACKNOWLEDGEMENTS

I would like to express my appreciation to all those who have helped in one way or another during the course of this study.

I am especially indebted to Dr. H. W. Buston for his kindly help and tolerant guidance throughout the duration of this work.

I would also like to thank Dr. J. Russell, Dr. J. L. Gay and many other staff and student colleagues for their willing and helpful discussion.

Finally, I would add thanks to Mrs. P. Tiranti and Mrs. S. Brown for their practical assistance, and Mr. A. Horne, who took the photographs.

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888 Biochem.
MED

30 July 1964

Dear Sir,

I am directed to inform you that you have been appointed as Examiner in conjunction with

Dr. B.E. Plunkett, Ph.D.,
Birkbeck College,
Malet Street,
London W.C.1.

for the thesis, a copy of which is enclosed, submitted by the following candidate for the Ph.D. Degree in the Faculty of Science as an Internal Student:

Name: D. Tyrrell (I.C.)

Thesis: 'The Influence of Carbon Dioxide on the Growth and Metabolism of Chaetomium Globosum'

I enclose the Instructions to Examiners for the Ph.D. Degree and would draw your attention to Sections 12 to 14, from which you will see that an oral examination should be held, unless the candidate is rejected without further test. I shall be glad if the Examiners will suggest a suitable time and place for the oral examination so that I may summon the candidate to attend.

The joint report of the Examiners should be written on the candidate's entry form which has been sent to your co-examiner.

Will you please return the thesis with the Examiners' report. I regret that I am no longer in a position to give permission to any Examiner to retain a copy of the thesis, in view of a scheme approved by the Senate with regard to the disposal of copies of theses after examination. This scheme makes no provision for the retention of theses by Examiners. Fewer copies are now submitted by candidates for the examination, and two of these copies must be bound, in accordance with the approved specification, before the conferment of the degree.

I enclose a copy of the Scheme of Payment to Examiners for Internal Students.

Yours faithfully,

James Henderson
Academic Registrar

Dr. H.W. Buston, D.Sc., A.R.C.S.,
Imperial College of Science and Technology,
South Kensington,
London S.W.7.

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INSTRUCTIONS TO EXAMINERS FOR PH.D. DEGREE FOR INTERNAL STUDENTS IN THE FACULTIES OF THEOLOGY, ARTS, LAWS, MUSIC, MEDICINE, SCIENCE, ENGINEERING AND ECONOMICS

1. Boards of Studies shall recommend the names of two Examiners for each candidate but in the case of the Ph.D. Degree in the Faculties of Arts and Economics Boards of Studies are empowered whenever they consider it desirable to recommend the appointment of three Examiners, such Examiners to take equal parts in the examination.

2. At least one of the Examiners shall, whenever practicable, have had experience in examining for the Ph.D. Degree of the University, and one Examiner shall, if possible, be the Teacher, or one of the Teachers, under whose supervision the research of which the results are embodied in the thesis has been carried out.

3. Boards of Studies may, if they so desire, recommend the names of two of the candidate's Teachers instead of the one Teacher mentioned above.

4. In cases where only two Examiners are recommended to act in the first instance, Boards of Studies shall also recommend the name of an additional Examiner to act only if called upon by the Principal after the Examiners who have acted in the first instance shall have reported to him formally that they are unable to arrive at an agreement; and the additional Examiner shall act jointly with the other Examiners and not as a Referee. Whenever practicable this additional Examiner shall have had experience in examining for the Ph.D. of the University.

5. When theses are sent to the Examiners, the Academic Registrar shall request them to acquaint themselves with the standard of the University, and inform them that copies of the theses submitted by candidates who have presented themselves for the examination previously are available for this purpose.

6. The standard of the Ph.D. Degree is definitely higher than that of the M.A. and M.Sc. Degrees in the same subject.

7. The thesis must form a distinct contribution to the knowledge of the subject and afford evidence of originality, shown either by the discovery of new facts or by the exercise of independent critical power.

8. The thesis must be satisfactory as regards literary presentation, and if not already published in an approved form, must be suitable for publication, either as submitted or in an abridged form.

9. The candidate must indicate how far the thesis embodies the result of his own research or observation, and in what respects his investigations appear to him to advance the study of his subject.

10. The Degree of Ph.D. will not be conferred upon a candidate unless the Examiners certify that the thesis is worthy of publication as a 'Work approved for the Degree of Doctor of Philosophy in the University of London'.

11. A candidate will not be permitted to submit as his thesis a thesis for which a Degree has been conferred on him in this or in any other University, but a candidate shall not be precluded from incorporating work which he has already submitted for a Degree in this or in any other University in a thesis covering a wider field, provided that he shall indicate on his entry form and also on his thesis any work which has been so incorporated.

12. After the Examiners have read the thesis, they may, if they think fit, and without further test, recommend that the candidate be rejected.

13. Except as provided in paragraph 12 the Examiners after reading the thesis shall examine the candidate orally and at their discretion by printed papers or practical examinations or by both methods on the subject of the thesis, and, if they see fit, on subjects relevant thereto; provided that a candidate for the Ph.D. Degree in the Faculty of Arts who has obtained the Degree of M.A. in the same subject in this University shall in any case be exempted from a written examination.

14. If the thesis is adequate but the candidate fails to satisfy the Examiners at the oral, practical or written examination held in connection therewith, the Examiners may recommend the Senate to permit the candidate to re-present the same thesis and submit to a further oral, practical or written examination within a period not exceeding 18 months specified by them, and the fee on re-entry, if the Senate adopt such recommendation, shall be half the normal fee.

15. If the thesis though inadequate, shall seem of sufficient merit to justify such action the Examiners may recommend the Senate to permit the candidate to re-present his thesis in a revised form within 18 months from the decision of the Senate with regard thereto; and the fee on re-entry, if the Senate adopt such recommendation, shall be half the normal fee. Examiners shall not, however, refer any thesis without submitting the candidate to an oral examination.

16. When a candidate for Ph.D. Degree in the field of Statistics has not a preliminary Degree in Statistics the Examiners are recommended to test the candidate's general knowledge of statistical method and theory outside the special field covered by his thesis.

17. Examiners are informed that it is not within their power to recommend the conferment of a Degree other than that for which the candidate has entered.

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(1) Masters' Degrees (excluding M.S. and M.D.S.), Ph.D., D.Mus.

<i>Setting Papers</i>	£ s. d.
For each paper set (fee divisible between the Examiners taking part)	10 0 0
[M.Sc. History and Philosophy of Science—Part I: A fixed fee of £45 is divisible between the External Examiners for their work in setting papers]	
Mathematics (including Mathematical Statistics) and Statistics papers, each ..	12 0 0
For transcribing the whole or the major part of a paper into an Oriental script for reproduction by photography	1 10 0
For modification of a paper for Overseas	15 0 0
Papers in connection with Practical Examinations: <i>see</i> Practical Examinations below.	

<i>Marking Scripts</i>	£ s. d.
For each of two markings or readings, for each script	7 6 0
At the D.Mus. Examination payment for three markings is allowed.	

Oral Examinations (and Practical Tests at M.A.)†

Masters' Degree Examinations:

Per candidate (fee payable for up to four orals on any one day)	3 10 0
If more than one Internal Examiner acts in addition to the External Examiner, £3 10s. 0d. per candidate is divisible among the Internal Examiners.	
At Internal M.A. and M.Sc. Psychology examinations, the fee for the oral covers work in respect of a practical (if held).	

Ph.D. Degree Examinations:

To each of two Examiners and to a third if called upon by the Principal, or to each of three Examiners appointed from more than one Faculty, per candidate (fee payable for up to four orals on any one day)	3 10 0
If three Examiners are appointed from the same Faculty to act in the first instance, the following fees will be payable:—	

Internal Examinations.—A fee of £7 per candidate shared equally between three teachers, or a fee of £3 10s. 0d. per candidate to the Examiner external to the University and a fee of £3 10s. 0d. per candidate shared equally between two teachers.

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Practical Examinations (and Oral Examinations at D.Mus.)

For each practical paper set (fee divisible between the Examiners taking part) ..	10 0 0
For a practical paper set to cover more than one day's practical at M.Sc. Examinations in Biochemistry	20 0 0
For attendance (including marking of candidates' work):—	
Whole day	7 0 0
Half-day	4 0 0
At Internal M.A. and M.Sc. Psychology examinations, work in respect of a practical is covered by the fee for the oral (and the above practical fees do not apply).	
If oral examinations for the Intermediate Examination in Music, B.Mus. and D.Mus. Examinations are held consecutively on the same day, payment will be made for the whole time for which Examiners are present, and not for separate periods for each examination.	
If an External or Staff Examiner resides outside the University radius of 30 miles his attendance, if required for a single examination period only on any day, is to be reckoned as attendance for a day.	

† For conducting oral examinations at Part I of the M.Sc. Examination in Agriculture (selected subject Poultry Science) for candidates registering in and after October 1961, a fee of 10s. per candidate (minimum fee £4 per day) will be payable to each of two Examiners for each subject.

<i>Theses and Dissertations, etc.,</i>	£	s.	d.
<u>Masters' Degree Examinations:</u>			
For assessing material submitted by a candidate,* to each of two Examiners and to a third if called upon by the Principal	7	0	0
<i>except in the following cases:—</i>			
The fee for reading a thesis at the following examinations is £5 to each of two Examiners:—			
Internal M.A. Philosophy			
Internal M.A. and M.Sc. Psychology (if candidate is examined by thesis, oral, three papers and a practical)			
The fee for reading a dissertation at the following examinations is £5 to each of two Examiners:—			
M.A. Education examinations consisting of four papers, dissertation and oral			
M.Vet.Med. [the fee for a thesis is £7 to each of two Examiners]			
The fee for reading a Report at the Internal M.Sc. (Eng.) Examination, for candidates proceeding to the Degree by method of written papers and submission of Report, is £4 to each of two Examiners.			
At the M.Sc. Examination in Geophysics, without thesis, a fee of £5 per candidate is payable to each of two Examiners to cover reading the dissertation, examination of course-work and attendance at oral examination.			
If three Examiners read a thesis or dissertation, etc., in the first instance, the fee for two Examiners is divisible between them if they are all teachers of the University, or the full fee is payable to the Examiner external to the University and half the prescribed fee to each of the two teachers.			
<u>Ph.D. Degree Examinations:</u>			
For examination of a thesis, to each of two Examiners and to a third if called upon by the Principal, or to each of three Examiners appointed from more than one Faculty.. .. .	10	0	0
If Examiner is candidate's Supervisor	7	0	0
If three Examiners are appointed from the same Faculty to act in the first instance, the following fees will be payable:—			
<i>Internal Examinations.</i> —A fee of £6 13s. 4d. to each of three teachers, including the Supervisor, or a fee of £10 to the Examiner external to the University and half the prescribed fee to each of two teachers [£3 10s. 0d. to the Supervisor and £5 to the other teacher].			
<i>External Examinations.</i> —A fee of £10 to each Examiner, provided that the appointment of the three Examiners has been specifically approved by the External Council after consideration of a special report of the appropriate Board of Studies.			
No fee shall be payable to a Teacher of the University for reading and reporting on a Thesis or Dissertation submitted in the joint names of the candidate and himself.			
For re-reading a thesis or dissertation submitted for a Ph.D. Examination and re-submitted for a Master's Examination or for re-reading a thesis or dissertation re-presented in connection with further tests in oral, written or practical examinations, the fees payable are half the fees prescribed for the first reading.			
For re-reading a thesis or dissertation submitted in a revised form, the fees payable are as for the first reading.			
<i>Design at M.A. (Architecture)</i> under old Regulations—For assessing material submitted by a candidate, to each of two Examiners and to a third if called upon by the Principal	7	0	0
If three Examiners act in the first instance, the fee for two Examiners is divisible between them.			
<i>Portfolio of Drawings</i> at Internal M.A. or M.Sc. Architecture under new Regulations, per candidate, to each of two Examiners	5	0	0
<i>Problem (at Mathematics)</i> (fee divisible between the Examiners taking part)	12	0	0
<i>Musical Exercise (D.Mus.)</i> (fee divisible between the Examiners taking part)	21	0	0
<i>Special ad hoc Qualifying Examination</i> —fee per candidate (fee divisible between the Examiners taking part)	10	0	0
For a qualifying examination, for the M.Sc. Examination in Botany, consisting of an oral examination only or of an oral examination together with one or more papers of an existing examination, fee divisible among the Examiners taking part in the oral examination	5	0	0

*To include 'an approved piece of textual and editorial work' at M.A. Examination in English and Education.

Chairmen's Fees								£	s.	d.
M.Th. (old Regulations) (Common)	..	..	..	..	..	..	..	15	0	0
M.A.:										
Classics (Internal and External)	..	..	..	..	..	..	..	15	0	0
Classical Chinese (Internal and External)			..	..	..	..	..	12	0	0
English—December (Common)	..	..	..	..	..	..	..	9	0	0
May (Common)	..	..	..	..	..	..	..	15	0	0
French (Internal and External)	..	..	..	..	..	..	..	18	0	0
Indo-Aryan (External)	..	..	..	..	..	..	..	15	0	0
LL.M. (Common)	..	..	..	..	..	..	..	25	0	0
M.Sc.:										
Agriculture (Poultry Science) (Internal)*	..	..	..	..	..	..	..	9	0	0
Biochemistry, to the Chairman of a Board of more than two Examiners*							..	9	0	0
Crystallography (Internal)*	..	..	..	..	..	..	..	9	0	0
Geophysics (Internal)*	..	..	..	..	..	..	..	12	0	0
History and Philosophy of Science (Common)			..	..	..	..	..	15	0	0
Microbiology (Internal)*	..	..	..	..	..	..	..	9	0	0
Radiation Biology and Radiation Physics (Internal)			..	..	..	..	..	12	0	0
M.A. and M.Sc. Mathematics under old Regulations (External)						..	..	[Under consideration]		
Meetings										
To each External or Staff Examiner, for attendance at each Meeting if summoned by the University (maximum fee £7 10s. 0d. per day)						..	..	2	10	0
No fee if held concurrently with a Practical Examination										
Minimum Fee to ad hoc, Staff or External Examiner			..	..	..	..	..	8	0	0

(2) D.D., D.Lit., LL.D., D.Sc., D.Sc.(Eng.), D.Sc.(Econ.)

To each Examiner who acts, inclusive fee, per candidate	..	..	..	..	..	..	..	15	15	0
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Travelling Expenses

For each occasion on which an Examiner is required by the University to travel a distance of more than 30 miles from his usual residence for Practical Examinations, Oral Examinations, or Examiners' Meetings, he may claim for journeys actually performed first-class return railway fare, together with the following allowances:—

For necessary absence from home not involving a night:

For a period of 5–10 consecutive hours, 15s. 0d.

For a period of more than 10 consecutive hours, £1 10s. 0d.

For each necessary period of absence up to 24 hours involving a night away from home, £3.

For journeys to or from Berwick or Carlisle or stations in Scotland or Northern Ireland, an allowance of £1 per journey, in addition to the subsistence allowance of £3.

An Examiner external to the University may claim fares and allowances as set out above for all necessary journeys actually performed from his usual residence (irrespective of the 30-mile limit).

July 1963

J. HOOD PHILLIPS
Secretary to the Senate

* Fees cover any External candidates examined by the Board.