INOCULUM-SIZE PHENOMENA IN ASPERGILLUS ORYZAE

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A THESIS

submitted to the University of Glasgow

for the

Degree of Doctor of Philosophy

in the Faculty of Science

University of Strathclyde,

Glasgow.

December, 1965.

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SUMMARY

Reports by a number of authors in widely differing fields of biological study have shown that various phenomena accompany a change in the size of an inoculum. Such phenomena have been shown in the present work to occur in cultures of *Aspergillus oryzae* under a variety of conditions. The importance of size of inoculum in growth, in metabolism of carbon and nitrogen and in respiration has been considered. The size of the inoculum chosen has been found to be a determinating factor in each one of the aspects of metabolism studied.

It has been shown in growth experiments, particularly with reference to maximum yield that deep cultures are influenced by inoculum size to a greater extent than are stationary cultures and that increase in mechanical stress enhances the effect of inoculum size. Large-inoculum cultures usually show a higher maximum yield than do small-inoculum cultures.

The effects of inoculum size on growth are generally

more pronounced when no trace elements are added. The effect of trace elements has been examined not only by using trace-element-poor chemicals, but by blocking trace elements with a chelating agent, removing them by a purification procedure or adding them individually or in combination. It has been shown that each individual trace element makes its own contribution to the inoculum-size effect and the resultant effect is a composite one depending upon the balance of trace elements in the substrate. A balance can be struck which can give a reversal of the usual effects of inoculum size, that is, smaller growth rate or maximum yield are obtained with large-inoculum cultures. Caramelization of the substrate results in a masking of the effects.

Aspergillus oryzae has been shown to produce substances inhibitory and stimulatory to its own growth. Stimulating substances are evident in large-inoculum cultures at all stages of growth and in small-inoculum cultures at late stages of growth. Inhibitory substances predominate in early stages of growth of small-inoculum cultures.

Since the differences between results for the growth experiments were sometimes small, statistical methods were applied to assess the validity of claims made.

The metabolism of carbon and nitrogen has been studied in this work. Consideration of the utilization of the carbon source and production of acids, together with incorporation and excretion of nitrogen has shown that large-inoculum cultures are generally more efficient than small-inoculum cultures at comparable mycelium contents. As the mycelium ages, it decreases in efficiency.

A number of factors influence the respiratory activity of Aspergillus oryzae. At late stages of growth, uptake of oxygen is reduced. Uptake of oxygen is reduced when the mycelium ages, or is subjected to a washing treatment, or is collected on a detergent-treated filter. Each individual component of the substrate has some influence on rate of uptake of oxygen. Small-inoculum cultures at all stages of growth capable of being collected and examined had the low rate of uptake found in late (but not in early) stages of growth of large-inoculum cultures.

" Various are the ways to change the state

Of plants, to Bud, to Graff t'Inoculate "

VIRGIL : Georgics. II, 103

Dryden's transl. 1697

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C O N T E N T S

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SECTION 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

11 PREAMBLE

111. General historical background

The English word 'inoculum' is derived from two Latin words, in, into and oculus, an eye, a bud. To inoculate meant originally to insert as a bud or graft. It is evident that in a horticultural context, the question of the size of the inoculum or material being inoculated or grafted would hardly arise.

The earliest usage of the word 'inoculum' in microbiology was in vaccination procedures. Even then, in 1722, small-pox was described as being inoculated 'upon' criminals. The association between a scarification technique and inoculation in the sense of grafting was obviously still strong. The usage of the term was then extended to cover the introduction of other antigenic material, even by the parenteral route, in order to produce antibodies. In this immunological setting, because the relationship between antibody production and size of inoculum was obscure, little interest developed in the

latter. Indeed interest in a phenomenon such as inoculum size could hardly arise while the inoculum was an unknown admixture of the organism desired with other unwanted organisms. Koch's systematization of microbiological techniques and his establishment of procedures designed to obtain and maintain pure cultures meant that at last the art of inoculation was given a scientific basis. At the same time, methods for estimating the size of an inoculum became more precise. For instance Pasteur and Liebig had obtained differing results in a similar experiment. Pasteur had obtained fermentation, Liebig no fermentation, yet each had used a 'pin-head' inoculum of yeast. Hilditch's explanation (see Schopfer, 1949) of the discrepancy is a likely one, i.e. that the results were due to a difference in size of inoculus because a French pin head is larger than a German!

It may be profitable to digress to consider the expected role of size of inoculum in the growth of a micro-organism such as a mould. If two flasks containing identical substrates are prepared, inoculated and incubated in the same way, identical growth patterns may be expected, regardless of the criterion chosen for study. If now one variable is introduced, namely size of inoculum (by using

a small inoculum in one instance and a large inoculum in the other) it is of interest to speculate as to how growth would be affected. It could be reasoned that the small inoculum would grow until it reached the size of the large inoculum and that from this point there would be no difference in the development of the two cultures. Apart from the extra time required for the small inoculum to reach the size of the large, the conclusion on this reasoning would be that the cultures would behave identically. Investigation of this phenomenon showed that this reasoning was not borne out experimentally, and further studies were indicated.

1.1.2. Reasons for the work and the approach to it

Inoculation is one of the most basic of all microbiological techniques, and the size of the inoculum is an aspect which must be considered. It is surprising in the circumstances that size of inoculum has been subjected to so little scientific scrutiny. Published work on size of inoculum has been done systematically with bacteria only, and that to only a limited extent.

Systematic work on size of inoculum to fungi has not been published until the recent series of which this work forms a part, although there are a number of references to the phenomenon in the literature. Aspergillus oryzae was chosen for this work as being an organism of some considerable industrial importance, and various aspects of its growth and metabolism were studied in relation to inoculum size. Among the factors studied were growth rate, maximum yield, carbon and nitrogen metabolism, trace element supply, self-produced inhibitors and stimulators, and respiration. It was thought that the first step in the investigation was to describe and characterize any phenomena resulting from a change in inoculum size in accordance with Virchow's dictum (cited by Holman, 1962) that "the study of the things caused must precede the study of the causes of things" and that has been attempted here. This was expected to open up avenues of exploration for other disciplines such as genetics and biochemistry. Such work, it was considered, would be of both fundamental and applied interest; fundamental because phenomena associated with a basic microbiological technique were being explored;

applied because cultures from different sizes of inoculum gave rise to end products differing in kind, amount or rate of production and some of these findings would possibly have industrial application.

12 REPORTS OF INOCULUM-SIZE PHENOMENA

Reports of size-of-inoculum phenomena are to be found as early as 1906, when Kuhn reported that increasing the number of bacterial cells in an inoculum reduced the lag period (Porter, 1946; Gunsalus, 1951). Influence of various factors on lag subsequently attracted considerable attention and Monod (1949) made a useful review of aspects of growth of bacterial cultures, including the lag phase. Lodge and Hinshelwood (1945) considered that a diffusible substance was produced during growth which was able to remove early lag in Bacterium lactis aerogenes. They stated "We should therefore expect the lag to depend upon the inoculum size: first, because some of the original medium is transferred with the inoculum, and, secondly, because larger numbers of organisms will more rapidly

build up the required concentration of the lag-reducing substance in the new medium. These expectations are confirmed." Lodge & Hinshelwood (1943) also considered that the lag should be less the greater the number of cells transferred in the inoculum, even when the amount of the old medium transferred was kept constant.

Considerable interest has been shown recently in the inter-relationship between size of inoculum and resistance. Such studies are of particular relevance to antibiotic treatment, since various severities of infection will result in various numbers of organisms to be treated. Allen and Nevdgh (1963) dealt with growth curves, lag and size of inoculum in growth of penicillin-resistant and penicillin-sensitive strains of Staphylococcus aureus. Rollers and Lehmicke (1960) studied experimental staphylococcal infection in mice, and described effects upon the bacterial populations of the individual organs by variations in the size of the inoculum. The range of inocula tested was from 10^2 to 10^5 organisms per dose. Although no population curves could be obtained because of the high, early mortality with large inocula, it was found that a critical number of staphylococci, injected intravenously, were necessary in order to be able to produce the characteristic

kidney population curves. Rochstein, Schnaper and Young (1962) considered the dependence on inoculum size of resistance or sensitivity of staphylococci and thought that variations in inoculum size in disc sensitivity testing was a likely source of discrepancies found. The same workers recommended a standard size of inoculum for such tests. Holmes, Rich and Bondeco (1960) also stressed that the size of inoculum used in assessing penicillin sensitivities of staphylococci by the 5-disc plate method was critical. Barber (1947) showed that the degree of *in vitro* resistance of staphylococci to penicillin could vary more than 500-fold according to the size of the inoculum used but that variation of results on altering inoculum size was a feature of resistant strains. Kaplan and Koch (1964) conclude with statistical support that the size of the inoculum is of particular importance when testing strains resistant to penicillin. Constant results irrespective of inoculum size were obtained with sensitive strains.

Various workers have noted that a certain threshold value for size of inoculum required to be reached before a small inoculum would grow at all. This was shown to be true in defined media for *Neisseria intracellularis* by

Gronsoulez (1945) and for Shigella sonnei by Nakamura and Hitsch (1961). Fredette and Forgo (1962) examined the ability of 7 fluid non-synthetic culture media to allow growth from very small inocula, and found that there was a range of ability to do so.

In general, it may be said that inoculum-size studies using bacteria have shown that the lag phase can be decreased with increase in inoculum size; that a certain minimal size of inoculum may be necessary to establish growth at all in a chemically defined medium, and that even non-synthetic media differ in their abilities to support growth of very small inocula.

In yeasts, effects due to size of the inoculum have been shown. Pfennig and Wilson (1960b) showed that the extent of the negative Pasteur effect in Saccharomyces carlsbergensis depended on the size of the inoculum in the culture from which the material had been taken. Since they are unicellular fungi, yeasts very readily lend themselves to studies on inoculum size in fungi, because increase in numbers or cell material can be followed by such rapid techniques as counting or estimation of turbidity. Several are, moreover, industrially important. A study of the interdependence of size and age of inoculum and of the

interdependence of size of inoculum and trace element supply was made with Geotrichum klaeckeri by MacKinnon (1964). He found that size of inoculum had an effect on the growth rate and maximum yield of that species. The trace elements supplied to the medium modified the effects considerably.

In moulds, studies on effects of inoculum size have to some extent been conditioned by the methodology available. Moreover, the growth of filamentous fungi is apical and not naturally accompanied by fission (see Pirt & Callow, 1960), and this has led to some confusion in analogies made with the kinetics of bacterial growth. Hawker et al. (1963) suggest that "the phase of unrestricted growth in liquid cultures (corresponding to the exponential phase of bacterial growth and to the linear phase of fungus growth on solid media) will be one of cubic growth". The implication here is that moulds do not pass through an exponential phase. Pirt and Callow (1960) however, showed that with unlimited amounts of all nutrients available, growth of the mould could follow the exponential law of bacterial growth and therefore the mould had a constant doubling time. Meyrath (1963) has made a plea for a more embracing terminology in growth kinetics of micro-organisms, so that terms used

may have equal meaning when applied to bacterial and fungal growth.

Inoculum size has been shown to be connected with the morphological characters of the organisms studied. Takahashi, Inada and Asai (1958) in studies on the effect of physical conditions on submerged mould cultures, reported on the effect of inoculum size on the shape of the pellet formed by Aspergillus niger under shaking conditions. Barthakur-Barua and Nickerson (1962) reported work on size-of-inoculum phenomena in Mucor rouxii and showed that heavily inoculated cultures incubated under nitrogen had a significant proportion of yeast-like cells amidst typically filamentous growth.

Other workers have shown that growth effects accompany a change in inoculum size. Kobayashi and Abuaiya (1962) showed that when rice leaves were inoculated by spore suspensions of Pyricularia oryzae, the number of lesions increased with the density of the spores.

Yates (1957) studied the influence of the inoculum on variability in comparative nutritional experiments with fungi, using Clavicens purpurea, and found that as the inoculum decreased, so variation among replicates increased.

The importance of standardization of the inoculum in nutritional experiments with fungi was stressed by Ward and Golotolo (1950). A low-temperature basidiomycete (taxonomic position unknown) was used and they also found (cf. Raber 1937) that variability between replicates was inversely related to the quantity of inoculum per culture flask over a certain range. It was emphasized that a standard procedure was necessary for the preparation of an inoculum.

Enzyme systems have also been shown to be affected by alteration in inoculum size. Moyrabh (1964b) has shown that in deep cultures of Aspergillus oryzae the rate of production of amylase is considerably increased in cultures from small inocula.

In general it may be said that there is good reason for considering the kinetic aspects of mould growth on the same plane as the kinetics of bacterial growth. Further, changes in size of inoculum in fungal culture have been shown to be accompanied by changes in growth characters, morphology and enzymatic constitution.

Interest in inoculum size phenomena has not been confined to bacteria, yeasts and moulds. Phelps (1935a, 1935b) studied the growth of Protozoa in pure culture, with

particular reference to the effect upon the growth curve of the age and size of the inoculum and different concentrations of nutrient materials. He found the 'initial stationary phase' of Glycogen to be independent of size of inoculum. Reichman (1962) found that the latent period of X-polyoma virus became shorter as the size of inoculum increased. Caplin (1963) studied the effect of initial size on growth of plant tissue cultures using Jerusalem artichokes. After 17 days' growth, he found that relative increase was inversely related to initial weight. He suggested two mechanisms to explain the fact that the large explants grew relatively less, namely, (a) production of a stalling factor and (b) a decrease in ratio of surface to volume.

In the work which follows, several aspects of growth, metabolism and respiration of Aspergillus oryzae are considered with particular reference to phenomena dependent on inoculum size. Additional literature is considered in the appropriate sections.

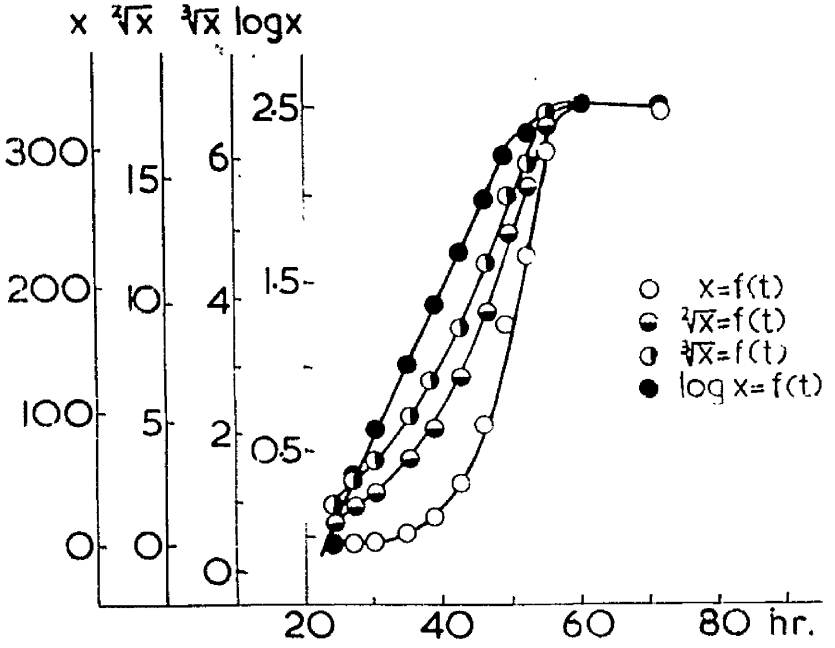


Fig.1 Growth curve of Aspergillus oryzae in deep (vibrating) culture plotted in four different ways. x = mg dry wt/100 ml; substrate A, starch. (Courtesy J.Meyrath from Antonie van Leeuwenhoek (1963) 29 57-78)

13 METHODS OF PRESENTATION OF INFORMATION

The graphical form in which results are conveyed must be chosen with care in order to present the findings of the work to the best advantage. Kahn (1959) described various graphical representations of algebraic formulae, indicated those of microbiological application and helped to establish a more precise terminology in an area where mathematics and microbiology coincided. Hinshelwood (1946) dealt with the kinetics of the bacterial cell and described the phases of growth through which a growing culture passed. These topics have been discussed more recently by Rance (1969). In the present work both exponential and linear phases are considered. As the exponential phase occurs before the linear phase (see Fig. 1) at a time when the mycelium content is low, large samples have to be taken. Deep cultures are more suitable for large-sampling procedures (see below) and the exponential curves given in this work are always for deep cultures. Hegrath (1963) has discussed the significance of the various vertical axes shown in Fig.1.

Some possibilities of presentation of linear curves are considered in Fig.2, in which the same information is

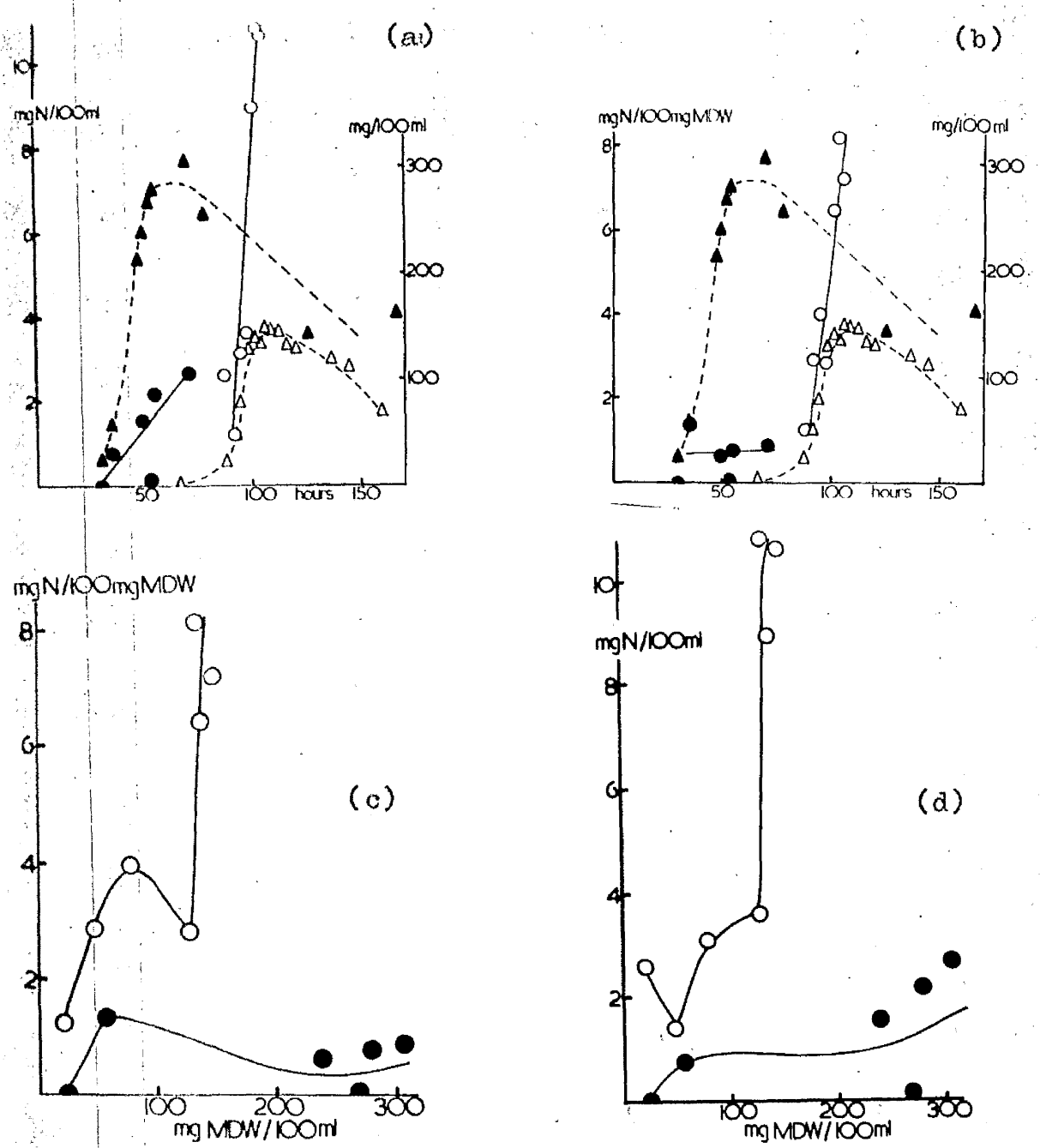


Fig.2 Organic nitrogen excreted by the mycelium in deep cultures of Aspergillus oryzae grown from large (● - ●) and small (○ - ○) inocula. The information is plotted in four different ways (a,b,c & d). The corresponding growth curves are included as broken lines in a and b for large (▲ - ▲) and small (△ - △) inocula, and the vertical scale on the right of the diagrams should be used for these curves. The material for these graphs has been reproduced or recalculated from Figs.7 and 36.

plotted in four different ways. The feature chosen is the organic nitrogen content of a culture of Aspergillus oryzae. This organic nitrogen is excreted by the mycelium into the surroundings. The extent of its excretion is considered for cultures grown from large as well as from small inocula and is shown as continuous lines (Fig.2 a,b,c and d). In the cases the growth curves of the same cultures (that is curves showing increase and decrease in mycelium content) have been included for comparison and are shown by broken lines (Fig.2 a and b).

The growth curves themselves (broken line in Fig.2 a and b) show one of the simplest forms of presentation of results. Weight is chosen for the y-axis and time for the x-axis. Weight is adjusted so as to indicate weight from a chosen volume of nutrient. From such a graph, the maximum yield in absolute terms is obtained from the height of the curve. The velocity of production of mycelium is indicated by the steepness of the slope. This is the type of presentation used for growth experiments throughout this work.

When production of a metabolite in the culture, such as organic nitrogen (i.e. nitrogen excreted), is plotted in the

same way, having the weight of nitrogen per unit volume for the y-axis and time for the x-axis, again the height of the curve shows maximum yield of the feature and the slope shows rate of production, both being in absolute terms. When this method of presentation is used to compare the production of the chosen feature by mycelium of cultures from large and small inocula however, the comparison is not the most advantageous. When the curves are laid alongside the corresponding growth curves (as in g) it can be seen that the production of organic-N by the mycelium is far from being directly proportional to the amount of the mycelium. Indeed the proportionality is inverse because the culture with a high yield of mycelium gives only a small amount of organic-N in the filtrate and the culture with a low yield of mycelium a large amount. When mycelium is being compared in terms of efficiency, it is necessary to relate the chosen feature to the weight of mycelium. This is readily accomplished by using the weight of nitrogen per unit of mycelium for the y-axis (as in h). Use of such a y-axis gives the relative or specific amount of organic-N present. Taken absolutely (as in g) the maximum excretion of organic-N by the small-inoculum culture is less than four times that of the large-. Taken

relatively, however, (as in b) it is over six times.

When such information is plotted (as in b) with time for the x-axis, it allows these comparisons to be made at various points of time. This however is not truly informative when dealing with cultures from large and small inocula, because these are at different stages of development and mycelial content at particular points of time, the large-inoculum culture usually being in the phase of measurable growth sooner. This means that there is always a 'displacement' in graphic representations of cultures in which time is chosen for the x-axis. For this reason, this form of graph has not been considered appropriate to these studies and instead, graphical forms with the y-axis just described, but with weight for the x-axis instead of time, have been substituted. An example of such an arrangement is shown in c, and any two points in vertical line represent the same weight of mycelium. In this way, the advantage of a y-axis which gives relative or specific values is combined with the advantage of an x-axis which allows comparison of cultures at points of equal content of mycelium. Graphs of this type have been used in the work on carbon and nitrogen metabolism (section 2) where the production of a particular metabolite is being followed.

There is a fourth possibility of arrangement of such graphs, namely combining the y-axis of g, with the x-axis of g. This has been done in d. Such an arrangement allows comparison of the two kinds of culture at any particular point of mycelium production because of the x-axis used. The ordinate at various stages of growth gives the amount of the feature being considered in absolute terms. The disadvantage of this method is that relative efficiencies cannot be compared so easily in this way at early stages of growth, where mycelium content and concentration of metabolites are small and this kind of graph has not been used in this work. This method of representation has, however, been found useful in a discussion on amylase production by the same organism (Meyrath, 1964c), and acid production (pH) by various species of Aspergillus (Meyrath & McIntosh, 1965b).

The difference in the information given when the y-axis is logarithmic instead of linear is exemplified in Fig.27 (p.102). When the y-axis is exponential, the slope of the line at any point shows the relative rate of increase (Fig.27a). When the y-axis is linear, the slope of the line at any point shows the absolute rate of increase (see Fisher, 1958).

It should be noted that where cultures from large and small inocula appear on the same graph, the large-inoculum cultures are represented by closed symbols, the small-inoculum cultures by open symbols.

14 STATISTICAL ASSESSMENT OF RESULTS

In the work which follows, quantitative results were obtained from three main kinds of experiment, namely, on oxygen uptake, on carbon and nitrogen estimation and on growth. In the first of these, the points showed very little scatter and yielded curves which were straight lines or almost such. This allowed rates to be calculated comparatively easily. The second of these yielded curves which were fairly distinct so that comparisons could be made without difficulty. The third group yielded a large number of curves and the difference between curves was sometimes small. Accordingly, a wide selection of part or all of these curves was subjected to statistical analysis.

The value of statistical analysis lies in the fact that an objective standard may be laid down to help to

decide whether an experiment is consistent with a hypothesis or not. The objective standard generally accepted in biological work is that the experiment is consistent with the hypothesis only when the figures in the result could not have occurred by chance alone more than once in twenty trials. As a chance of one in twenty has been chosen to indicate significance, so a chance of one in one hundred is usually accepted as highly significant (Holman 1962). To eliminate confusion, when the word "significant" and its derivatives are used in this work, they are used with their full statistical connotation. When results could not occur by chance more than once in 20 times (i.e. 5% limits) they are termed significant, more than once in 100 times (i.e. 1% limits), highly significant and more than once in 1000 times (i.e. 0.1% limits), very highly significant. When theoretical values are cited from statistical tables (as in the Tables of the various Figures) without further qualification, the 'significant' or '5%' level is implied. Where higher levels of significance are considered, the appropriate percentage is quoted alongside.

Diagrams were constructed from the results prior to statistical analysis. One good reason for so doing is

described by Fisher (1943). "diagrams ... are invaluable as adjuncts to statistical analysis ... ... when used as a preliminary to the analysis, they serve to draw the attention of the experimenter to the salient features of his data and frequently ensure that he does not overlook some unexpected relationship. In doing this a diagram will generally suggest the form of analysis most appropriate to the data" The diagrams given in this work were constructed from points representing mean values of experimental readings, and the statistical treatment to be followed was deduced from a study of the graph.

When the theoretical relationship between two values x and y is unknown, it is common practice to develop an empirical mathematical equation relating them (Saunders and Fleming, 1957). The simplest relationship is a straight line when the relation between x and y is

$$y = bx + a$$

where a is the intercept on the y-axis and b the slope of the line. Graphs with inflections may be represented by equations with x in increasingly higher powers.

There were good reasons for attempting to obtain a straight line relationship if possible. A straight line has the merit of simplicity. Furthermore, where a

straight line relationship obtains, determination of the slope of the line is made easy. Accordingly, where a whole curve did not seem likely to yield a straight line relationship, a part of the curve which was more nearly a straight line was chosen for statistical examination.

A series of points such as those obtained over all or part of the curves in Section 2 inevitably shows a scatter due to random error. While the most likely straight line corresponding to these points can be drawn roughly by eye, the best-fitting straight line can be determined more accurately by regression analysis.

In applying this method, it is assumed that one variable is free from error. In the case of the curves given in Section 2, the time measurements were assumed to be correct. The basis of the calculation of the line of best fit is the method of least squares. By this method, a line is considered to be the best fit for a collection of points if the sum of the squares of the distances (measured in the y direction) of all the points from the line is a minimum - that is, is smaller than the corresponding sum for any other line. This line (whose equation has the form $y = bx + c$) is called the regression line and b, the regression coefficient, gives the slope of

the line. It is to be noted that the choice of a best line even by this method, is arbitrary and that if another more useful average line were found, there is no reason why it should not be used instead (Vessels 1962).

Although the standard deviation, the square root of the variance, is a familiar function for measuring the scatter of data, it is the variance that is amenable to mathematical treatment. If a process has a number of factors contributing to the variance of the final measurement, this total variance is equal to the sum of the variances of the individual factors when these are statistically independent (Volk 1958). With this in mind, use was made of the fact that for most points on the curves, triplicate readings existed. While the mean of these readings showed a scatter due to departure from linearity, the readings themselves showed a scatter about the mean for any one point. This meant that two variances could be assessed, namely, that due to variation between groups and that due to error within groups. In turn, variation between groups could be broken down into that due to regression and that due to departure from regression. The variance due to error within groups was made the basis

for estimating the acceptability of the variance due to departure from regression by calculating an appropriate 'F' value. The straight line relationship was accepted or rejected on the basis of whether the 'F' value fell within statistically acceptable limits.

When acceptable straight lines were obtained, their slopes could be compared by comparing the difference between two regression coefficients with the standard error by means of a 't' test. The standard error was found by taking the square root of the sum of the variances of the two coefficients. An electric calculating machine was used to perform these calculations.

The 't' test for significance was not only used to compare the regression coefficients of two lines. It was also used to estimate the significance of differences between single points for which repeated measurements were held. The calculations were of a standard nature and highly repetitive. They were thus very suitable for treatment by computer and a 'Sirius' computer was made available for the purpose.

The curves for no trace element addition in Fig.9 (● - ● and ⊙ - ⊙) exemplify the growth curves which had to be examined for inoculum-size effects in stationary

cultures. It will be noticed that the curves have somewhat similar shapes, but that they are displaced in time. At the commencement of measurement, the small-inoculum culture takes four days to reach the content of mycelium possessed by the large-inoculum culture in two days. If from that point the two curves were of precisely the same shape it might be expected that over the ascending part of the curve, at any given point of time, the large-inoculum culture would have a greater content of mycelium. Such curves could, however, be superimposed and, in this work, no effect due to size of inoculum would be claimed in such a case. If, however, the rates of growth differed from each other, this would be held to constitute evidence for an inoculum-size effect. The rate of growth would be obtained from the slope of the curve. Also, a difference in maximum yield would be taken to indicate an effect of inoculum size, as in Fig.7. Further, conclusions about inoculum-size effects were sometimes made from single points of the growth curves after due allowance had been made for the time differential operating at the beginning of the curves.

When curves from one size of inoculum were being compared, as, for example, the closed symbols of Fig.9,

it was permissible to compare points at the same time. This would, for example, allow conclusions to be drawn about the effect of a particular trace element on cultures derived from one size of inoculum. It could not by itself be termed an inoculum-size effect.

SECTION 2

GROWTH EXPERIMENTS

2 GROWTH EXPERIMENTS

21 INTRODUCTION

Because a bacterial culture grows by the reproduction of individual organisms giving rise, in many species, to a uniform turbidity, it has been possible to estimate growth of the culture by enumeration of individuals or assessment of turbidity. A mould culture on the contrary, grows by extension of mycelium, with or without septation, and so is not open to growth estimation by an enumeration method. An enumeration method is not, in any case, free from difficulties. For instance Gunsalus (1951) defined growth as an increase in protoplasm and pointed out the dangers of relying on cell counts as an index of growth. Moreover, as turbidity of a mould culture is seldom uniform, turbidity methods cannot be used for assessment.

Several other methods for growth estimation of mould cultures have been tried and are described by, for example, Hanker (1950). Cochrane (1958) showed the inadequacy of measuring the rate of growth in a tube or the diameter of a colony, in order to estimate growth rate. Cochrane suggested that total cellular nitrogen deserved wider use

as a determinant for growth. Since incorporation of nitrogen is a metabolic activity, it might be expected to rise and fall with other aspects of metabolic activity. Later (section 3) experimental evidence is given in support of this contention. For this and other reasons dealt with more fully in the section on nitrogen metabolism, total cellular nitrogen was not used as a measure of growth.

It was necessary, of course, to choose some standard of growth. Weight had the merit of wide acceptance in other biological work and, bearing in mind the limitations of the other methods mentioned, was considered the best criterion available in fungal physiology. This view was supported by the finding that when weight was used as a basis for growth determination and plotted against time, the resultant curve showed the kind of orderly progression of growth to be expected over a considerable range of culture development, that is, unrestricted (or exponential) growth.

Having decided upon weight estimation for growth determination on both practical and theoretical grounds, the method to be employed for the cultivation of the organism required consideration. Three types of culture

in common use were considered, namely stationary culture, shake culture and deep culture.

Shake cultures were put on a scientific basis by Kluyver and Perquin (1933) and have been widely used. Apart from a reliable shaking device, no more provision than that for stationary cultures need be made. They have the advantage of homogeneity of content, making sampling and testing easier. Although not used in the present work, shake culture techniques using the same organism have been reported elsewhere (Meyrath 1963).

A shaking method is to be avoided in work on inoculum size because of possible variation in pellet size in shake cultures. A large inoculum tends to produce a large number of small pellets but a small inoculum tends to produce a small number of large pellets. The differing physical properties of the resultant cultures could make it difficult to interpret the results in terms of inoculum size.

When stationary culture techniques are employed, an adequate oxygen supply can be provided only by a considerable surface area. Under these conditions, the mycelium grows as a felt on the surface and the culture is unsuited for representative sampling. These difficulties

are overcome by using, as here, a number of small (50-ml) conical flasks, each containing 10 or 20 ml substrate. When a sample is required, several of these flasks may be taken together.

Deep culture methods are extensively used in industrial processes, in pilot-plant fermentations and in laboratory-scale experiments. A comparison of stationary with deep methods was made and a comparison of deep cultures under different conditions was made also.

The inoculum for a culture of a mould such as Aspergillus oryzae may consist of mycelium or conidia. Mycelium may be inoculated in a variety of ways. Gunderson (1962) examined three methods using as inoculum mycelium from each of three fungi, namely Trichoderma viride, Fomitopsis pinicola and Fomes annosus. The mycelium was inoculated in one of the following forms: a, suspensions; b, hand-cut squares; c, standard discs. He found only the last of these to be of value. Conidia however are much more easily washed than is mycelium, and because of the applicability of a counting procedure, their numbers are readily estimated. Conidia were therefore used throughout these experiments. Meyrath (1963) has already shown that inoculum-size phenomena occur with an inoculum

of mycelium.

An important role has for long been ascribed to trace elements in inoculum-size phenomena. Since the time of Gopping (1929) a 'carry-over' theory has been predominant; increased growth rate and maximum yield consequent upon using a large inoculum have been credited to a larger transfer of trace elements with the inoculum. Some experimental evidence (Hayrath, 1963) discounts this explanation in the case of Aspergillus oryzae, and the role of trace elements was therefore investigated rather more fully here.

Preliminary work by Hayrath (1962a) showed the presence of self-stimulating substances in cultures of A. oryzae. This work was expanded to include self-inhibitory as well as self-stimulatory substances, in both stationary and deep cultures.

22 MATERIALS AND METHODS

221. Glassware

All glassware was acid-washed to reduce trace element contamination. This was done by steeping in 7% HCl, usually overnight. The glassware was then rinsed 5 times in tap water and finally twice in de-ionized or glass-distilled water collected in an acid-washed flask.

222. Organism and Inoculum

Aspergillus oryzae was the organism used throughout the growth experiments except for one occasion when Aspergillus niger NRRL 3 was used (Fig.6). The strain of A. oryzae came from the Department of Agricultural Bacteriology and Fermentation, Swiss Federal Institute of Technology, Zurich, is strain No.754 in the collection of the University of Strathclyde and has been reported on in the literature (e.g. Meyrath, 1963; Meyrath & McIntosh, 1963b; McIntosh & Meyrath, 1963c).

In all experiments, conidia were used as inoculum.

At first the conidia were produced on malt wort slopes in 1 oz. screw cap bottles. As the bottles were small, a large number had to be used. Moreover, the degree of sporulation was often limited. There were two probable reasons for this, (a) lack of oxygen in the sealed bottle and (b) variability and unsuitability of malt wort. Accordingly, a sporulation substrate of the following composition was used (substrate H):

Starch BDH Lab. reagent	40g
$(NH_4)_2SO_4$	6g
$MgSO_4 \cdot 7H_2O$	0.5g
$FeCl_3 \cdot 6H_2O$	0.005g
KH_2PO_4	34g
Na_2HPO_4	35.5g

water to 1000 ml.

This substrate has a high content of both phosphate and nitrogen. It was distributed in 25-ml amounts in 10-oz. flat bottles with cotton-wool plugs, and sterilized by autoclaving at 15 lb. for 15 min. When stored at room temperature for some time it formed a gel and hence was steamed and cooled immediately before use to allow distribution of the inoculum. After inoculation the bottles were incubated

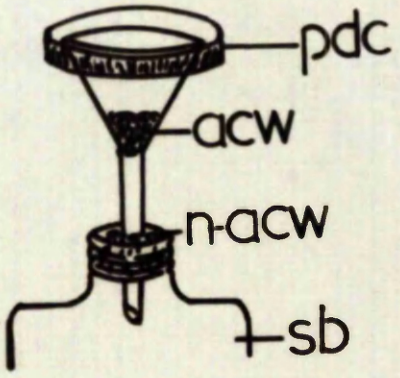


Fig.3 Arrangement for collection of conidia.

p d c	petri-dish cover
a c w	absorbent cotton wool
n - a c w	non-absorbent cotton wool
s b	sterile bottle

at 25° in a flat position. The necessity for oxygen for conidial production was evident in such cultures, for sporulation was usually heavier near the plug than distant from it. Heavy conidial production was obtained in 10 days.

The substrate was drained and after replacing the cotton-wool plug with a sterile screw cap, the mycelial felt was agitated with 2 or 3 successive 25-ml volumes of sterile water. The liquid on each occasion was filtered through absorbent cotton wool. This cotton wool was held in a filter funnel covered by a petri-dish lid; the funnel was inserted into a sterile bottle (Fig.3) which could afterwards be centrifuged directly and the whole unit was sterilized before use. Most of the conidia passed through the wool, while mycelial fragments were retained. When the latter clogged the filter, conidia retained could be released by pressure on the cotton wool mass with a sterile rod. A few washings with sterile water through the cotton wool also helped to free further conidia. The suspension of conidia was then centrifuged at 2,500 rpm for 5 min and re-suspended in 20 ml sterile de-ionized or distilled water, the procedure being followed three times. The conidia were then re-suspended in sterile water and counted in a

Table A

Composition of Substrates

All substrates contained (g/l) : carbohydrate (starch 10; if otherwise, kind and concentration are specified); $(NH_4)_2SO_4$, 1 ; $MgSO_4 \cdot 7H_2O$, 0.3. The other components are shown below.

	A	A_1	A_3	A_4
		g/l		
Na_2HPO_4	4.73	4.73	0.46	4.73
KH_2PO_4	4.54	4.54	9.08	4.54
		mg/l		
$FeCl_3 \cdot 6H_2O$	15.0	15.0	15.0	15.0
$CuSO_4 \cdot 5H_2O$	0.5	-	-	0.05
$MnSO_4 \cdot 4H_2O$	0.5	-	-	0.05
$ZnSO_4 \cdot 7H_2O$	5.0	-	-	0.5
$CaCl_2 \cdot 6H_2O$	10.0	-	-	1.0

haemocytometer. The conidial suspension was stored in the refrigerator at 2° and was usually used within 6 weeks of preparation. Viability was checked frequently by making ten-fold dilutions of the stock suspension until an estimated 50 to 100 conidia per ml were present. Three 1-ml samples were introduced into sterile petri dishes and mixed with malt wort agar. When this had set, the plates were incubated for three days when the colonies could be counted by the naked eye. It was assumed that at the concentration of this viability test, the distribution of conidia was such that they had no influence on each other. The viability of the conidia was usually above 50%.

223. Substrates

The terminology of earlier publications (e.g. Meyrath, 1963), has been followed and is summarized in Table A. Variations from those substrates are noted in the figures or in the text. All chemicals were of analytical reagent quality. Starch or glucose was the carbohydrate used. When starch was used, a suspension of it in cold water was

poured into a boiling solution of the remaining constituents; boiling was maintained for a few minutes and the cooled solution was made up to volume. Starch was autoclaved together with the minerals. Glucose, when included, was autoclaved separately from the mineral solution and mixed aseptically afterwards in order to avoid caramelization. If trace elements were added, they were mixed with the minerals before sterilization. Sterilization was by autoclaving at 15 lb/sq.in. (122°) for 15 min.

2.4. Supplementation of culture by culture filtrate or ethylenediaminetetraacetic acid (EDTA)

Filtrate from deep cultures was sterilized by passing through a 5/3 sintered glass filter. Filtrate from stationary cultures was subjected to discontinuous sterilization by treatment with free-flowing steam for 20-min periods on each of three successive days.

When tests were carried out to reveal the presence of stimulating and inhibiting substances, 0.5 ml of a particular culture filtrate was added to each one of a set of flasks at the same time as the medium was inoculated with

conidia; this is described later under inoculation of stationary cultures. When the culture was to be supplemented by EDTA an appropriate amount was measured out from a 3% w/v aqueous solution of EDTA and added to the mineral solution before making it up to volume and sterilising. Subsequent procedure was as for unsupplemented substrate.

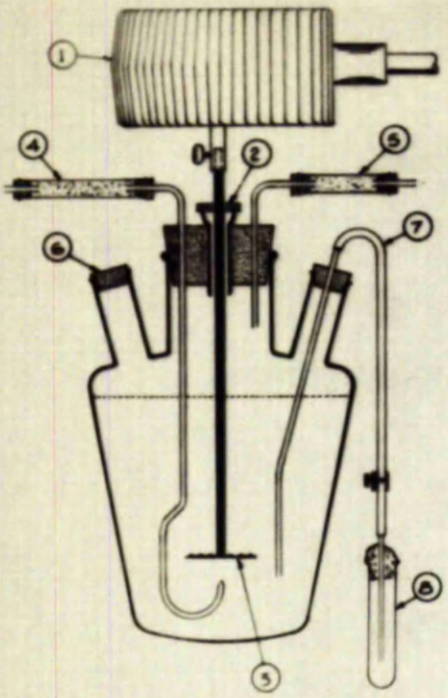
225. Purification of glucose in substrate by $Al(OH)_3$

When purification of the glucose was desired a procedure similar to that of Pan & Johnson (1948) was used. This is summarized as follows. An excess of glucose was weighed out, dissolved and made up to a volume giving a higher concentration than was required (e.g. for a final volume of 2.5 l, 125g instead of 100g glucose made up to 1 litre). Aluminium sulphate - $Al_2(SO_4)_3 \cdot xH_2O$ (x = approximately 18) - at a concentration of 2.5 g/l was added, dissolved and the liquid adjusted to the desired pH value with KOH. The suspension was stirred for 6 h by magnetic stirrer then centrifuged at 2000 rpm for 15 min. The supernatant

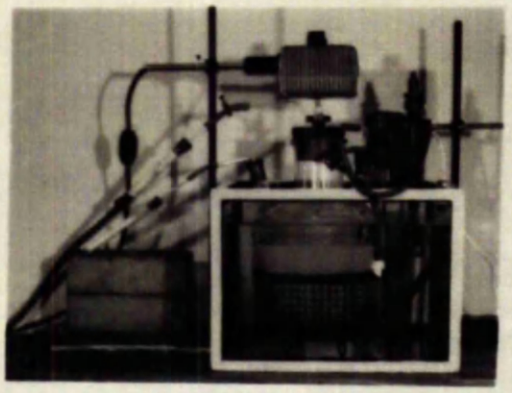
was poured off and diluted to the final concentration required.

226. Caramelization of substrate

In one instance this took place inadvertently and the effects were noted (Fig.22, p. 89). On another occasion, caramelization was undertaken deliberately; the results are recorded in Fig.23. It had been suspected that the unintentional caramelization (Fig.22) was due to traces of $Al(OH)_3$ left during sterilization. Accordingly, this was deliberately provided (Fig.23) in the form of aluminium sulphate and KOH. One hundredth of the amount used in the normal precipitation method was added - 0.0025g $Al_2(SO_4)_3 \cdot xH_2O$ (x = approximately 18) and 0.02 ml KOH. The glucose was subjected to the usual autoclaving procedure in the presence of this material. No browning was evident, so the flasks were autoclaved again - for an additional 30 min at 15 lb/sq.in. Although only little browning had taken place, the substrate was then used and the results noted. (Fig.23).



(a)



(b)

Fig.4 Apparatus for deep culture of Aspergillus oryzae. Agitation is brought about by a vertical vibrator.

Fig.a (Courtesy J.Meyrath from Experientia (1964) 20, 235) shows (1) vibrator; (2) sealing gland; (3) vibrator disc; (4) air inlet filter; (5) air outlet filter; (6) inoculation orifice; (7) sampling siphon; (8) sterile tube to prevent contamination of the siphon.

Fig.b shows the apparatus as used.

227. Apparatus for stationary and deep cultures

Stationary cultures: these were grown at 25° in 50-ml conical flasks closed with plugs of non-absorbent cotton wool.

Deep cultures: the vibrating stirrer method of propagation used for deep cultures has been described recently (Meyrath 1964a) and some of its applications recorded (Meyrath 1964b). The diagrammatic representation of the assembly has been reproduced together with a photograph of the apparatus in Fig.4. The outstanding advantage of this arrangement as compared with other deep culture methods is the reduced risk of contamination. This stems from the use of a vibrator instead of a revolving stirrer. The latter requires a gland to allow free rotation of the stirrer. The necessary looseness of such a gland presents a risk of contamination overcome only by a complex sealing mechanism. In contrast, in the arrangement described, the agitation is performed by a vertical vibrator rather than by a rotating stirrer. The vibrator is held in place by a tightly-fitting diaphragm of resilient material. This material gives the freedom of

movement necessary and at the same time forms a tight seal with the vertical vibrating rod. The vibrator could be adjusted and amplitudes of 2 mm (high mechanical stress) or 1 mm (low mechanical stress) were used.

The flask was triple-necked with a capacity of 6 l and a working volume of 5 l (Fig.4). Air from a compressor pump was passed through a sterile cotton-wool filter and into the culture at a rate of approximately 500 ml/min. The outlet was so arranged that the air was released deep in the flask, in an upward direction, below the centre of the vibrator plate. This gave an efficient distribution of air and permitted an efficient transfer of oxygen to the culture; at the same time clogging of the air inlet by mycelium was minimized.

The flask was held at a temperature of 25° in a water-bath.

228. Inoculation of stationary and deep cultures.

Stationary cultures: An inoculum of 10^7, 10^3 or 10 conidia per 20 ml substrate (for large, small and extremely small inocula respectively), in a volume of 1 ml sterile water for each 20 ml substrate was used. Additive, if included, was in a volume of 1 ml per 20 ml substrate. The combined additive and inoculum was added to the bulk substrate and the whole distributed in 50-ml flasks as required. Amounts of 20 ml were usually used when large amounts of filtrate were required, as when testing filtrate against other cultures, but in all other instances, 10-ml amounts were used. Up to 12 different sets of 24 flasks were tested concurrently, usually half of these sets being large-inoculum and half small-inoculum cultures. It should be noted that by any procedure, the content of conidia was approximately as follows: large inoculum, 5×10^7/100 ml; small inoculum, 5×10^3/100 ml; extremely small inoculum, 50/100 ml.

Deep cultures: These were always grown in 5-l amounts of substrate and the conidia used for inoculation were suspended in sterile water to give a 50-ml inoculum. In all cases, with one exception, the inoculum consisted of

2×10^7 conidia per 100 ml substrate (large inoculum) or 4×10^5 conidia per 100 ml substrate (small inoculum). The exception was the deep culture used to test filtrate for stimulating and inhibiting substances (p.102). In this case the inoculum was the same as that for the stationary cultures used to test the filtrates - 5×10^7 conidia per 100 ml substrate (large inoculum) or 5×10^3 conidia per 100 ml substrate (small inoculum).

229. Collection of mycelium from stationary and deep cultures

For reasons adduced elsewhere (p.132), assessment of growth was made by weight estimation at various intervals of time. This involved collecting the mycelium, drying it and weighing the pellets of dried mycelium. It was important to collect the mycelium at times which suited the stage of growth of the culture rather than at arbitrary pre-determined intervals. For example where a culture gave a higher growth rate and entered the phase of linear growth sooner than another culture, such differences required to be reflected in the times of collection of the mycelium samples.

Stationary cultures: Sets of 24 flasks were employed and at each sampling point a set of three flasks was taken.

allowing 3 sampling points in a series of results. The yield of mycelium was estimated for each flask separately except at early stages of growth where the amount of mycelium was low, in which case the contents of the 3 flasks were bulked.

The contents of the flasks to be estimated were poured into a piece of nylon or cellulose acetate fabric covering a Buchner funnel. The funnel was inserted tightly into a suction flask attached to a water-pump. If the filtrate was to be examined, it was commonly collected by adding to the funnel a short rubber tubing extension which led into a test tube in the suction flask. The filtrate was removed before further washing of the mycelium. The mycelium still adhering to the flask in which the mycelium had been grown was removed by rubbing, using a glass rod with an end covered with rubber tubing. Three rinsings of de-ionised water were used to assist in the transfer of all mycelium from flask to filter square. The mycelium was then pressed firmly with a rubber bung, rinsing the mycelium between pressings with de-ionised water. After the last pressing, the mycelium was formed into a small pellet. Residual water was pressed out and the pellet placed on filter paper in a petri dish. The

pellets were dried at 106° overnight, after which time and temperature constant weight was assumed. The individual pellets were cooled in a desiccator and weighed. From the figure obtained for the dry weight of material from a set of three flasks, the dry weight per 100 ml was obtained.

This procedure is an improvement on that using tared filter paper to retain the mycelium (see Cochrane, 1958) in that the mycelium can be pressed repeatedly without danger of rupturing the retaining material. Thus a large number of tests can be performed simply, without the accumulation of a filter paper for each pellet, and the weighing of the filter paper is avoided.

Deep cultures: Since deep cultures were grown in 3-l batches as described, a large volume of material was available for sampling. Samples were withdrawn by syphon. The slight positive pressure in the fermentation flask could be increased temporarily by blocking the air exit and if the syphon tube exit was then opened, the pressure inside was sufficient to start the syphon. The first 50 ml was discarded and the sample was then taken. After sampling the syphon was again sealed and its end placed in a tube of alcohol. Care was taken to avoid back syphoning

because it was likely to introduce contamination into the flask.

At early stages of growth when the mycelial content was low, very large samples (up to 500 ml) were taken. The size of the sample was progressively reduced as the mycelial content increased until 10-ml samples only were required. When the sample was of an easily manageable amount, it was taken in triplicate. The samples were then collected, washed, pressed, dried and weighed in the manner described for stationary cultures.

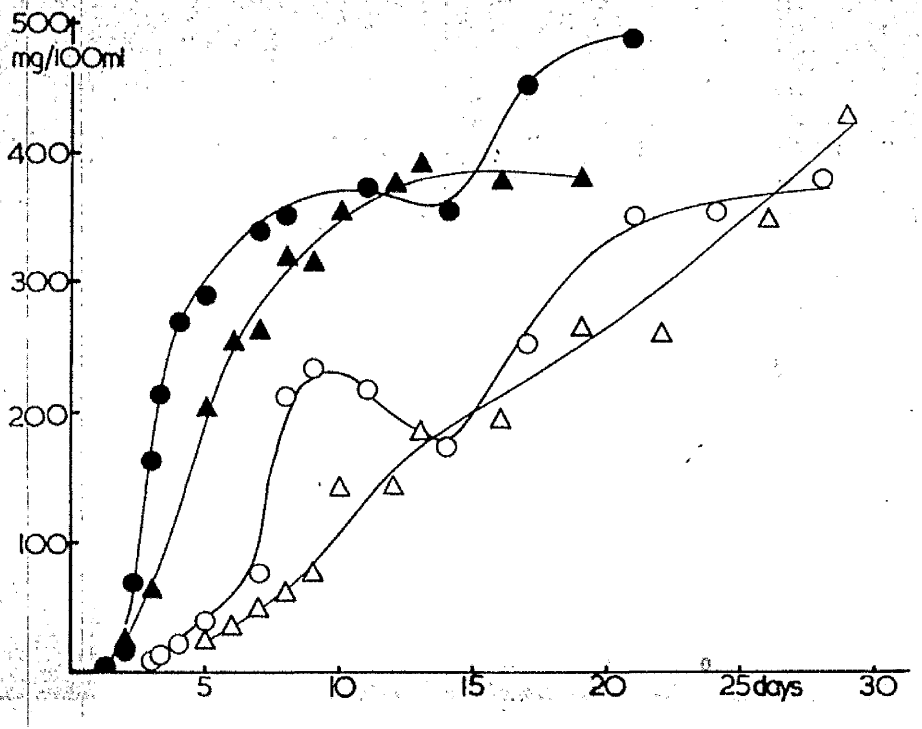


Fig.5 Growth of Aspergillus oryzae in stationary culture. Large-inoculum (●-●) and small-inoculum (○-○) cultures were grown in the presence of trace elements. Large-inoculum (▲-▲) and small-inoculum (△-△) cultures were also grown in the absence of trace elements.

Substrate: The substrate without trace elements was A_3 40g/l glucose except that the Fe content was 0.5 mg/l. The substrate with trace elements contained additionally per l: Cu, 0.05 mg; Mn, 0.05 mg; Zn, 0.01 mg.

Table of Fig.5

Statistical analysis

Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
● , samples 1-6	101.4 ±2(15.64)	1.32	3.84		
○ , samples 1-7	39.15 ±2(6.70)	0.87	3.11		
Comparison of these slopes				3.66	2.26 difference significant
▲ , samples 1-5	unacceptable	6.27	4.35		
▲ , samples 1-4	59.05 ±2(2.86)	0.80	5.79		
△ , 13 samples	15.94 ±2(0.81)	0.42	>2.18		
Comparison of these slopes				14.53	<4.32 (0.15) difference very highly sig.
△ , 13 samples	as before				
○ , samples 1-7	as before				
Comparison of these slopes				3.44	<2.13 difference sig.

Table of Fig.5 continued

	Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
○	13 samples	unacceptable	2.36	< 2.24		
○	13 samples except for samples 6, 7 & 8	16.11 ±2(0.82)	1.47	2.48		
△	13 samples	as before				
	Comparison of these slopes				0.15	>2.09 difference not sig.
▲	samples 1-4	as before				
●	samples 1-6	as before				
	Comparison of these slopes				2.66	2.45 difference sig.

Samples compared		't' actual	't' theoretical		significance
● and ▲ ;	5 day	6.45	4.60	(1%)	highly sig.
● and ▲ ;	7 day	3.65	2.78		significant
○ and △ ;	9 day	3.02	2.78		significant

23 RESULTS

231. Influence of a range of conditions

The first experiments described consider the effect of inoculum size under a range of conditions such as presence or absence of trace elements, stationary or deep culture, high or low mechanical stress and change of species of organism.

Fig.5 shows the pattern of growth in stationary cultures of Aspergillus oryzae. The stepwise increase in yield for cultures from both large and small inocula in presence of trace elements no longer held in their absence. This stepwise increase is reminiscent of diauxic effects already described (see Monod 1949) in which the effect is explained by, for example, a change-over in the constituent of the substrate being utilized for energy. This explanation is not valid here where glucose is the sole source of energy.

In order to obtain information on an inoculum-size effect, the growth rates at early stages were compared. The first six samples of the curve from the large-inoculum culture in presence of trace elements (● - ●) could be

represented by a straight line, as could the first seven samples of the small-inoculum culture curve (○-○). A comparison of the slopes of these two lines showed a significant difference between them (Table of Fig.5). We may infer, then, that Fig.5 shows an effect of size of inoculum in stationary cultures at an early stage in so far as the rate of growth of the small-inoculum culture was smaller than that of a large-inoculum culture in the presence of trace elements. The inference is limited because of the strong inflection of the small-inoculum curve. A similar comparison was undertaken for the cultures grown in the absence of trace elements. Although the first five samples for the large-inoculum culture curve (▲-▲) did not yield an acceptable straight line, the first four samples did so. All thirteen samples for the small-inoculum cultures in absence of trace elements yielded an acceptable straight line also (△-△). The difference between these lines was very highly significant indicating an effect of inoculum size. Again the inference was limited, this time because of the wide difference in range covered by the lines taken.

It was thought to be of some importance to examine the significance of the divergence between the curves for

the two large-inoculum cultures and the divergence between the curves for the two small-inoculum cultures. In the latter case, addition of trace elements resulted in a pronounced hump in the curve (Fig.5, O - O).

On statistical examination of the small-inoculum cultures, it was found that although an acceptable straight line could be fitted to the entire curve for the culture without trace elements (△-△, Table of Fig.5), this could not be done to all points of the curve showing the hump (O - O), perhaps because of it. It was however possible to fit a straight line to the first seven samples for this curve (O - O, Table of Fig.5), covering the range to the maximum for the hump. The slope of this line differed significantly from that of the regression line for all the samples of the small-inoculum culture without trace elements (△-△), giving a partial indication of the significance of the hump. To confirm this, 't' tests were applied to samples taken at the same time in the region of the hump. With small-inoculum cultures (O and △), the divergence was significant at 9 days, the maximum point of the hump, as is shown in the Table of Fig.5. The clearest indication of the significance of the hump came from the fact that although all 13 samples of the

culture concerned (O - O, Fig.5) did not yield a satisfactory straight line, a satisfactory straight line relationship was obtained when the samples of the hump (samples 6, 7 and 8) were omitted (Table of Fig.5). The slope of this line did not differ significantly from that of the curve without trace elements (Table of Fig.5). The probable indication is that initial rapid growth is followed by a slight partial autolysis which in turn is followed by renewed growth.

The slopes of the early parts of the curves for large-inoculum cultures were also compared and found to differ significantly (▲- ▲ and ● - ●, Table of Fig.5). Comparison between individual points on these curves was also made on those occasions on which samples had been taken at the same time. When 't' tests were applied to these cultures (Fig.5, ● and ▲), the divergence was found to be highly significant at 5 days and significant at 7 days (Table of Fig.5). An explanation similar to that advanced for small-inoculum culbures may account for this comparable, if less marked, phenomenon.

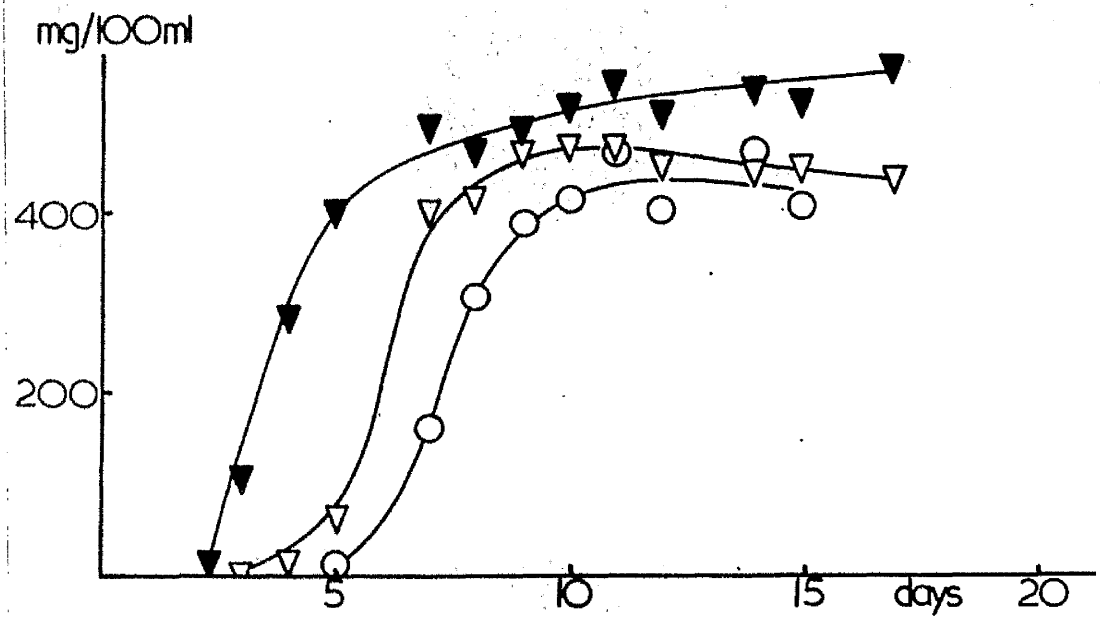


Fig.6 Growth of Aspergillus niger in stationary culture from large (▼-▼), small (▽-▽) and extremely small (○-○) inocula in a substrate without added trace elements.

Substrate: A_3 40g/l glucose except that there was an Fe content of 0.5 mg/l.

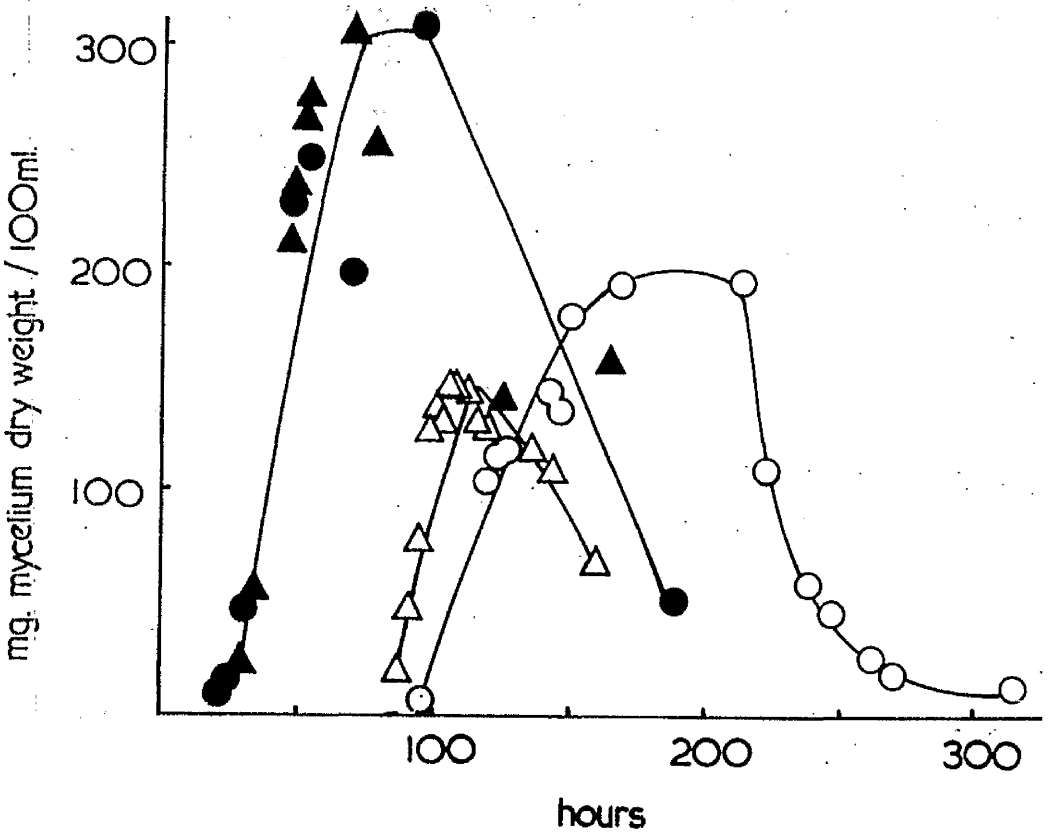


Fig.7 Growth of Aspergillus oryzae in deep culture. Large-inoculum cultures were grown under low (● - ●) and high (▲ - ▲) mechanical stress. Small-inoculum cultures were also grown under low (○ - ○) and high (△ - △) mechanical stress.

Substrate: A_4

Tables of Figs.6 and 7.

Table of Fig.6

Statistical analysis

	Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
▼,	samples 7-13 (last seven)	5.35 ±2(2.57)	2.77	3.03		
▽,	samples 6-12 (last seven)	-4.6 ±2(0.97)	0.1	3.03		
	Comparison of these slopes				3.63 difference significant	2.23

Table of Fig.7

Statistical analysis

	Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
● ,	samples 1-7	4.08 ±2(0.93)	not calculated: single observations only			
○ ,	samples 1-8	2.38 ±2(0.31)				
	Comparison of these slopes				1.73 difference not significant	>2.18

Although the work reported here was confined almost entirely to Aspergillus oryzae, Fig.6 shows the result of an examination of the growth properties of stationary cultures of Aspergillus niger as part of the investigations designed to test the universality of the occurrence of effects of inoculum size. The experiments were carried out in a substrate without added trace elements. It is to be noted that there was little effect on growth rate in the linear phase, whichever of three sizes of inoculum was used. When an 'extremely small' inoculum was used, A. niger did not, under these conditions, give the 'reversal' effect reported for A. oryzae by Meyrath (1963). The last seven points of the curves for large - (▼-▼) and small- (▽-▽) inoculum cultures could be represented by straight lines in each case (Table of Fig.6). When these straight lines were compared, they were found to be significantly different in slope at this late phase of growth, indicating that the large-inoculum culture was growing at a slow rate whereas the small-inoculum culture was autolysing at a slow rate.

When deep culture methods were used, the rate of growth in the linear phase is seen to be almost the same for cultures from large and small inocula (Fig.7). The

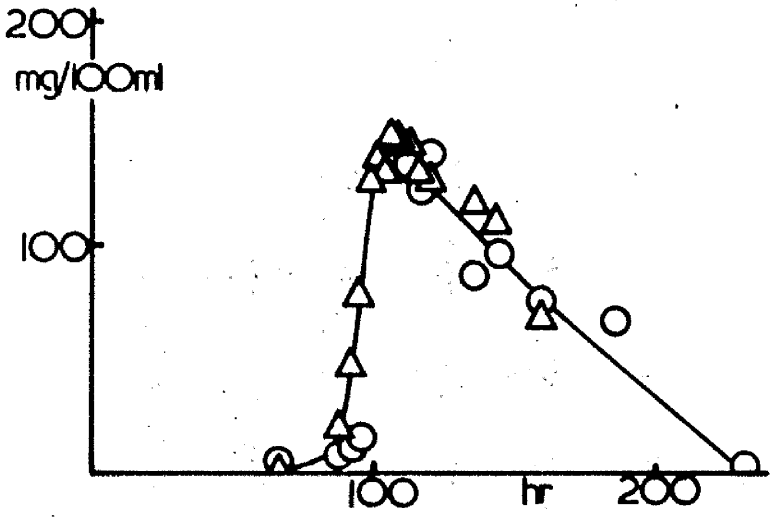


Fig.8 Growth of Aspergillus oryzae in replicate experiments at high mechanical stress in deep cultures from small inocula (O - O and △ - △). One set (△ - △) has been taken from Fig.7.

Substrate: A_4

difference in slope between cultures from large (● - ●) and small (○ - ○) inocula at low mechanical stress is not statistically significant (Table of Fig.7). The difference in maximum yield is, however, very marked. The maximum yield for large-inoculum cultures was almost twice as great as that for small-inoculum cultures.

The same figure (Fig.7) also shows the effect of mechanical stress on deep cultures. Again, maximum yield is the factor of greatest interest. In large-inoculum cultures (▲- ▲ and ● - ●) there is little apparent difference in maximum yield due to difference in mechanical stress applied. With small-inoculum cultures (△- △ and ○ - ○) however, an increase in mechanical stress resulted in a decrease in maximum yield. Combining these deductions, it may be said that an effect of inoculum size with respect to maximum yield was present under both conditions of mechanical stress. At high mechanical stress however, this effect of inoculum size was more pronounced.

Fig.8 is included to show how high reproducibility in these experiments could be. The curves shown are for duplicate experiments performed at different times with small-inoculum deep cultures under high mechanical stress. The points are very close throughout and a single line

suffices to describe both cultures.

The experiments described above indicate that size-of-inoculum phenomena of varying kinds and degrees may be found in a wide variety of conditions. Since an influence due to trace elements had been shown, and this had already been noticed elsewhere (Meyrath, 1963), it was decided to examine the influence of trace elements in more detail.

232. Influence of trace elements and chelating agents

It has been long recognised that trace amounts of heavy metals play an essential part in fungal physiology, although their function or status was not at first understood. Foster (1949) described the developments which led to a better understanding of their significance and showed that the early view was that their action (in small doses) was merely stimulatory; that is, it was assumed that in their complete absence there would still be growth in the basal substrate.

Steinberg (see Foster, 1949) among others showed, however, that when traces of these metals were rigorously excluded, negligible growth took place, but that upon

adding traces of, for example, Zn, increase in growth was inordinately large. For instance in a highly purified substrate only 1 mg dry wt/100 ml A. niger was obtained. Addition of trace amounts of Zn led to a 230,900 per cent increase in growth (Steinberg, 1934). Again, a two-fold increase in dry weight was caused by 1 part per thousand million of Zn (Steinberg, 1935). Only on the assumption that these trace elements are essential rather than simply stimulatory can those facts be explained.

Trace elements are essential because they are components of co-enzymes or enzyme systems. Morphology, cultural characters, sporulation, pigmentation, physiology and biochemical reactions may be influenced by a change in trace element composition or concentration. The main trace elements are Fe Cu Mn and Zn. Others such as molybdenum, vanadium and cobalt have been mentioned and as they are required in even smaller amounts than the main trace elements, Nicholas (1963a) has suggested that they might be called "ultramicronutrients" Mo, for example (required for nitrate reduction; see Nicholas, 1963b), is essential for A. niger to the extent of only 0.1 part per billion (Nicholas, 1952). Nicholas (1952) also showed that vanadium did not replace molybdenum, illustrating the

specificity often found.

Fe is a constituent of respiratory enzymes; catalase, cytochrome complexes, ferrichromes and haem pigment all contain it. The requirement for Fe by fungi is slightly greater than that for most trace elements, 0.1 - 0.3 ppm being necessary (Nicholas, 1963a). Aspergillin, the black pigment in spores of A. niger, contains 0.25% Fe and addition of Fe increases sporulation (see Nicholas, 1963a). The maximum formation of certain metabolites such as streptomycin and penicillin requires more Fe than is required for maximal growth (Cochrane, 1958).

Ca is a major nutrient for plants but its importance in the growth of fungi, in which it is required only in micronutrient amounts, has only recently been recognised. A number of workers have shown that it stimulates yeast growth (see Morris, 1958). Species in 19 genera, including representatives of all the major groups of fungi and of the genus Streptomyces, have been found to give increased dry weight on addition of Ca (see Cochrane, 1958). Unlike the other elements mentioned, it is not a heavy metal, and it is uncertain whether it has a specific effect or whether it simply protects against certain monovalent cations such as H, Na or K. The effects of Ca, according to

Nicholas (1963a), "have not been disentangled from those of pH". However Akabori and others in 1954, Oikawa and Maeda in 1957 and Oikawa in 1959 (cited by Steiner, 1960) showed that crystalline taka-amylase of A. oryzae contained one atom of Ca per molecule, firmly bound. Moreover, Ca may be required for the formation of proteases, a suggestion put forward in work on Staphylococcus pyogenes by Shooter & Wyatt (1955). The same authors showed that while Ca and Mg each stimulated growth by themselves, they were more effective jointly.

Mn has been shown to be essential in all genera of fungi studied thus far, in a concentration of 0.005 - 0.01 ppm (Nicholas, 1963a). Nicholas (1963a) also points out that it is essential for sporulation in a number of fungi (e.g. A. niger) and that it can take the place of magnesium in a number of reactions in which ATP is involved.

Cu is inhibitory to many micro-organisms at low concentration and has been used widely as a bactericide, fungicide and algicide, but is nevertheless required by fungi for both growth and sporulation at a concentration of about 0.01 - 0.1 ppm (Cochrane, 1958). It is a constituent of a number of enzymes - e.g. polyphenol oxidase. Nicholas (1963a) from his wide experience has found "that the

amounts of copper used in several of the recommended media for micro-organisms are often unnecessarily high, and in some at toxic concentrations". On the contrary, some organisms can withstand very high concentrations. Ferrobacillus, a bacterium deriving its energy from the oxidation of Fe^{++} to Fe^{+++}, tolerates concentrations of Cu as high as 2000 mg/l (Heyrath & Allan, unpublished).

Zn was found essential for growth of A. niger (Hawker, 1950). A large number of metabolic effects result from Zn deficiency, the major of which are listed by Cochrane (1958). The optimal range is reported as 0.001 to 0.5 ppm (Cochrane, 1958).

Consideration of the role of trace elements in inoculum-size phenomena is important because increased growth rate and maximum yield in a culture consequent upon using a large inoculum have been widely explained on the "transfer" principle. This credits the result to the carry-over or transfer of a large number of nutrients along with a large inoculum (e.g. MacLeod, 1959a, 1959b). There is now, however, sufficient evidence to make the "transfer" explanation questionable. It was shown directly by Meyrath (1963) that the transfer of trace elements with a large inoculum of conidia of Aspergillus oryzae was

insufficient to cause an effect on small-inoculum cultures in substrates poor in trace elements. From other experiments (Meyrath, unpublished) it was obvious that transfer of organic growth factors was not an explanation for the phenomena because when media containing yeast extract, malt extract or peptone were used for large-inoculum cultures, it hardly affected their growth rate as compared with cultures well provided with trace elements.

The influence of trace elements on size-of-inoculum phenomena is considered in the work which follows (see also McIntosh & Meyrath, 1963b, 1964b). Since chelating agents have so important a bearing on the operation of trace elements, consideration is also given to them.

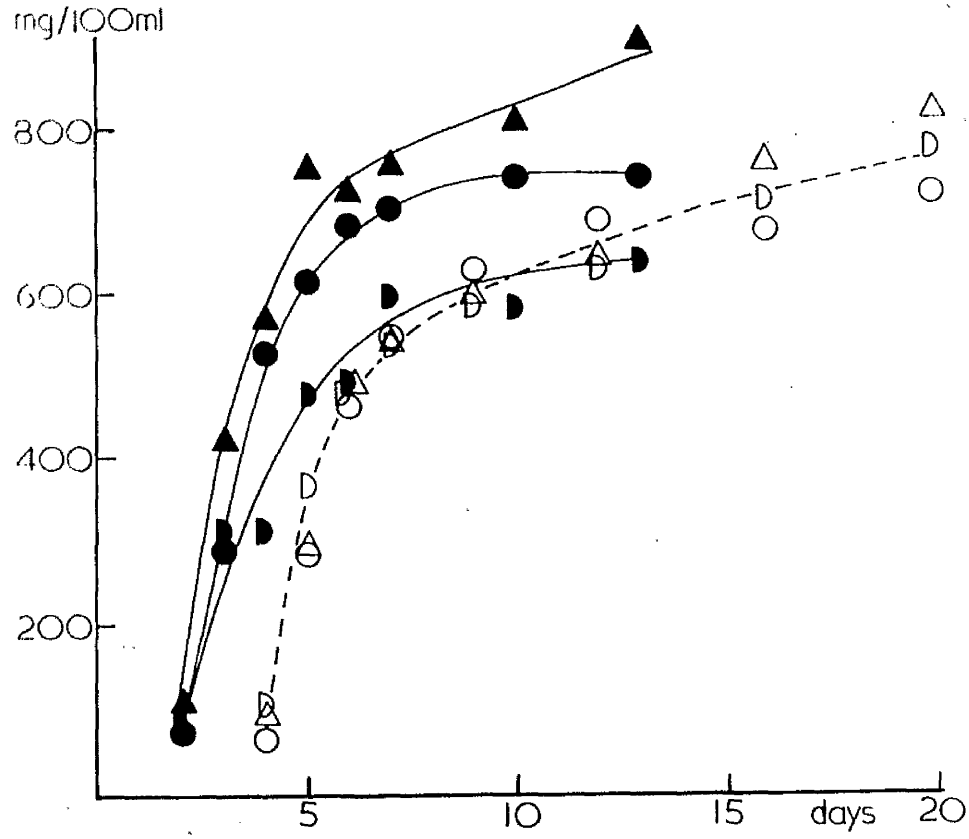


Fig.9 Effect of individual trace elements on growth of stationary cultures of Aspergillus oryzae from large and small inocula.

Large-inoculum cultures	small-inoculum cultures	trace element addition
◉ - ◉	○ - ○	none
▲ - △	△ - △	Ca as 10mg/l $CaCl_2 \cdot 6H_2O$
D - D	D - D	Cu as 0.5mg/l $CuSO_4 \cdot 5H_2O$

Small-inoculum culture results are represented by a single broken line.

Substrate: A_1 40g/l glucose

Table of Fig.9

Statistical analysis

Range	Regression coefficient	'F' actual	'F' theoretical	'r' actual	'r' theoretical
◗ . samples 1-5	93.89 ±2(14.08)	9.76	5.26		
D . samples 1-3	unacceptable	14.75	5.99		

Samples compared	't' actual	't' theoretical	significance
△ and ○ ; 9 day (5th samples)	1.10	2.78	not sig.
○ and D ; 9 day (5th samples)	1.40	2.78	not sig.
△ and ○ ; 12 day (6th samples)	3.3	2.78	sig.
○ and D ; 12 day (6th samples)	2.80	2.78	sig. *
△ and ○ ; 16 day (7th samples)	2.39	2.78	not sig.
○ and D ; 16 day (7th samples)	1.23	2.78	not sig.
△ and ○ ; 20 day (8th samples)	3.84	2.78	sig.
○ and D ; 20 day (8th samples)	1.59	3.18	not sig.
▲ and ● ; 10 day (7th samples)	3.49	2.78	sig.
● and ◗ ; 10 day (7th samples)	9.65	8.61(0.1%)	v.highly sig.
▲ and ● ; 13 day (8th samples)	6.17	4.60(1%)	highly sig.
● and ◗ ; 13 day (8th samples)	3.30	2.78	sig.

* note borderline value

2321 Effect of individual trace elements

The result of adding individual trace elements to a basal substrate without trace elements except for Fe, is shown in Fig.9. On comparing large-inoculum with small-inoculum cultures, the greatest divergence in growth rates (as shown by the slopes of the lines) appeared to occur when Cu was added. The difference, however, was not of the 'usual' order in which growth rate was higher for cultures from large inocula (see Meyrath, 1963). Indeed there seemed to be a 'reversal' of this finding, since growth rate was higher in the culture from a small inoculum. This reversal will be established if it can be shown that the slope of the line for the large-inoculum culture is significantly less than that for the small-inoculum culture. When this was attempted statistically, it was found that although the first five samples of the large-inoculum culture (● - ●) gave an acceptable straight line relationship, the first three samples of the small-inoculum culture (D - D) failed to do so (Table of Fig.9). An inoculum-size effect cannot therefore be confirmed statistically in respect of rate of growth since the kinetics of the two cultures are not comparable. The

regression coefficient for the line calculated from the first three samples of the small-inoculum culture (D - D) by the method of least squares was 187.33. Although this was rejected because of the 'F' value, the rejection appeared to be due to the fact that variation within the groups was so small. If accepted as an 'estimated' value, however, it indicates a reversal effect because it has almost twice the value of the slope for the first five points of the large-inoculum culture (● - ●), the value of which slope is 93.89. Fig.9 additionally contains useful information about the action of Ca and of Cu on large- and on small-inoculum cultures. Cultures from each size of inoculum are now considered separately.

Small-inoculum cultures (open symbols) were hardly affected by addition of Ca or Cu. Over the first half of the growth curve (first four samples of each), the mycelial dry weights frequently coincided (Fig.9). Over the second half of the growth curve (5th, 6th, 7th and 8th samples) there were slight divergences in the curves. The yields for cultures from small inocula with Ca and with Cu added were compared with the yields of the culture containing no additions, at the different points of this second half of the growth curve, namely at 9, 12, 16 and

20 days' growth (see Table of Fig.9). Addition of Ca resulted in alternate non-significance and significance in difference from the control culture (△and○, Table of Fig.9). Addition of Cu resulted in a non-significant difference in three out of four instances, and a significant (borderline) difference in only one instance, (○ and D, Table of Fig.9). Bearing in mind that over the first half of the growth curve the points usually lie very close together, the general conclusion may be drawn that Ca or Cu have little effect on small-inoculum cultures.

In large-inoculum cultures, Ca raised and Cu lowered the maximum yield of the cultures. The stimulation by Ca was significant at 10 days' and highly significant at 13 days' growth. The inhibition by Cu was very highly significant at 10 days' and significant at 13 days' growth, (Table of Fig.9).

If neither Ca nor Cu influences the growth of small-inoculum cultures, the fact that Ca stimulates large-inoculum cultures will result in a tendency towards a positive (or 'normal') effect of inoculum size. Similarly, the fact that Cu inhibits large-inoculum cultures will result in a tendency towards a negative (or 'reversal') effect of inoculum size.

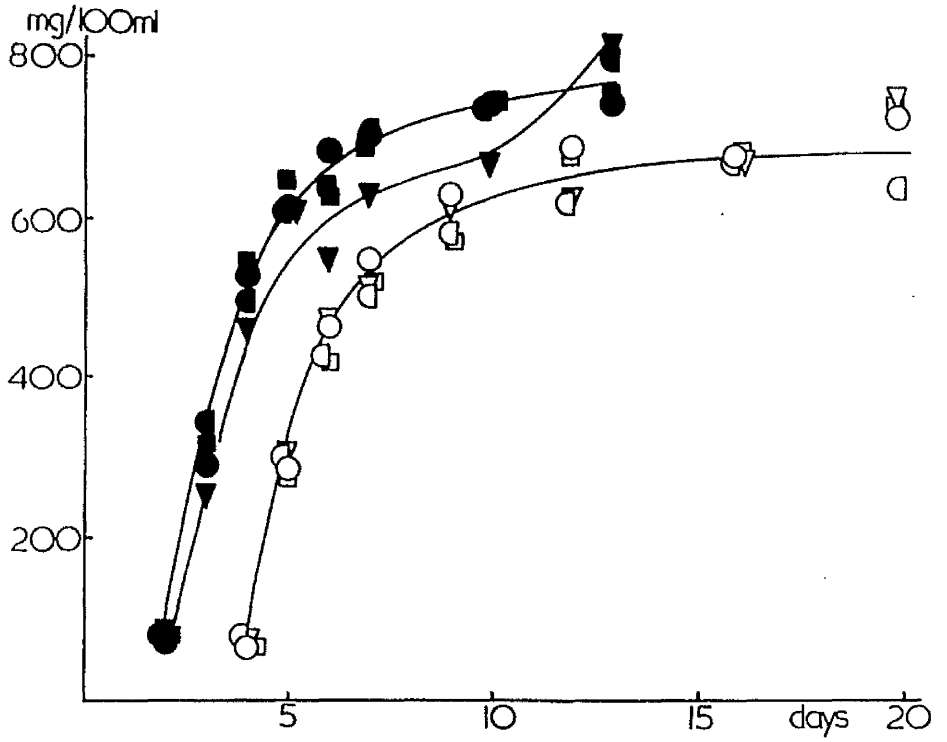


Fig.10 Effect of individual trace elements on growth of stationary cultures of Aspergillus oryzae from large and small inocula. The conditions were the same as those of Fig.9, and the control results (no trace elements) are repeated from that Figure.

large-inoculum cultures	small-inoculum cultures	trace element addition
● - ●	○ - ○	none
■ - ■	□ - □	Fe as 15mg/l $FeCl_3 \cdot 6H_2O$
▼ - ▼	▽ - ▽	Mn as 0.5mg/l $MnSO_4 \cdot 4H_2O$
◖ - ◖	◖ - ◖	Zn as 0.5mg/l $ZnSO_4 \cdot 7H_2O$

Substrate: A_1 40g/l glucose.

Table of Fig.10

Statistical analysis

samples compared		't' actual	't' theoretical	signif-icance
● and ■ ;	7 day (6th samples)	0.24	2.78	not sig.
● and ▼ ;	7 day (6th samples)	1.07	2.78	not sig.
● and ◀ ;	7 day (6th samples)	0.10	2.78	not sig.
● and ■ ;	10 day (7th samples)	0.03	2.78	not sig.
● and ▼ ;	10 day (7th samples)	1.73	2.78	not sig.
● and ◀ ;	10 day (7th samples)	0.17	2.78	not sig.
● and ■ ;	13 day (8th samples)	0.37	3.18	not sig.
● and ▼ ;	13 day (8th samples)	1.15	2.78	not sig.
● and ◀ ;	13 day (8th samples)	0.86	2.78	not sig.

Moreover, if Ca enhances the usual effect of inoculum size and Cu enhances the reversal effect, it may be anticipated that when both are present together, a reduction of effect of inoculum size whether normal or reversal, would be likely to take place.

The experiments of Fig.10 were performed under the same conditions as the preceding experiment but using Fe, Mn and Zn as trace elements. Again small-inoculum cultures were hardly affected. With large-inoculum cultures there was an apparent effect of Mn (▼-▼) and this effect was examined statistically at the last three points of sampling, by comparing the result with that for a corresponding culture without trace element addition. A similar statistical comparison was made at the same stages of growth for the other two trace elements added. (Table of Fig.10). The higher 't' values obtained when Mn was used ('t' = 1.07, 1.73, 1.15) as compared with other trace elements indicates that it has a more stimulatory effect than the other trace elements tested but since these values in no case reach the level of significance, no effect due to inoculum size is claimed under these conditions.

Although the curve for a culture with Fe as additive is given in Fig.10 (squares), the effect of Fe could not be

deduced clearly since Fe was present in the basal substrate (as in the cultures represented in Fig.9) and there was therefore no variant without Fe.

2322 Effect of phosphates

Phosphorus is not a trace element. In fact, large amounts of phosphorus are present in cells of moulds and yeasts (Hawker, 1950). It is necessary for phosphorylation in the breakdown of carbohydrate and as an essential constituent of nucleic acids and phospholipids. Phosphorus in the form of phosphate must therefore be included in all defined media. The salts of sodium and potassium phosphate served as a convenient buffer to give the pH value required. Since phosphates act as complexing agents and thus influence the availability of trace elements, they are considered here. As a preliminary to experiments on the addition of various trace elements, the influence of various concentrations of phosphate on the effects of inoculum size in stationary culture was examined.

The effect of three concentrations of phosphate on growth rate and maximum yield of cultures from large and small inocula is shown in Fig.11. On examination of the

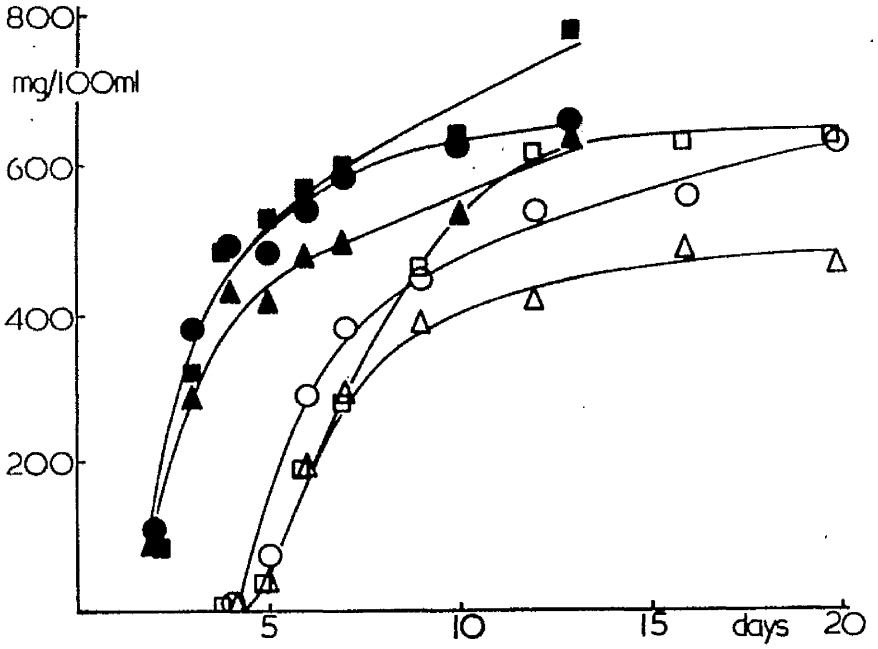


Fig.11 Effect of various concentrations of phosphate on growth of stationary cultures of Aspergillus oryzae from large and small inocula.

Large-inoculum cultures	small-inoculum cultures	concentration of total phosphate in substrate
⊕ - ⊗	○ - ○	M/5
△ - △	△ - △	M/15
⊡ - ⊡	□ - □	M/45

Substrate: A_3 40g/l glucose except that M/5, M/15 or M/45 total phosphate was used instead of M/7.5.

Table of Fig.11

Statistical analysis

Samples compared	't' actual	't' theoretical	significance
▲ , 13 day (last sample) and △ , 16 day (penultimate sample)	8.94	8.61 (0.1%)	very highly significant
▲ , 13 day (last sample) and △ , 20 day (last sample)	5.12	4.60 (1%)	highly significant

difference between curves from experiments using large and small inocula it can be seen that there was no striking effect due to variation in phosphate content within the range given. Of the three concentrations examined, M/15 phosphate appeared to give a marginally greater differentiation between large-inoculum and small-inoculum cultures than did the other concentrations. When the last-recorded weight of the large-inoculum culture was compared statistically with the last two recorded weights of the small-inoculum culture (when the maximum stationary phase had been reached) the differentiation when M/15 phosphate was used was found to vary from very highly significant to highly significant (Table of Fig.11). M/15 was, in any case, the concentration of phosphates in substrates A, A_1 and A_4 and was the concentration retained for most experiments.

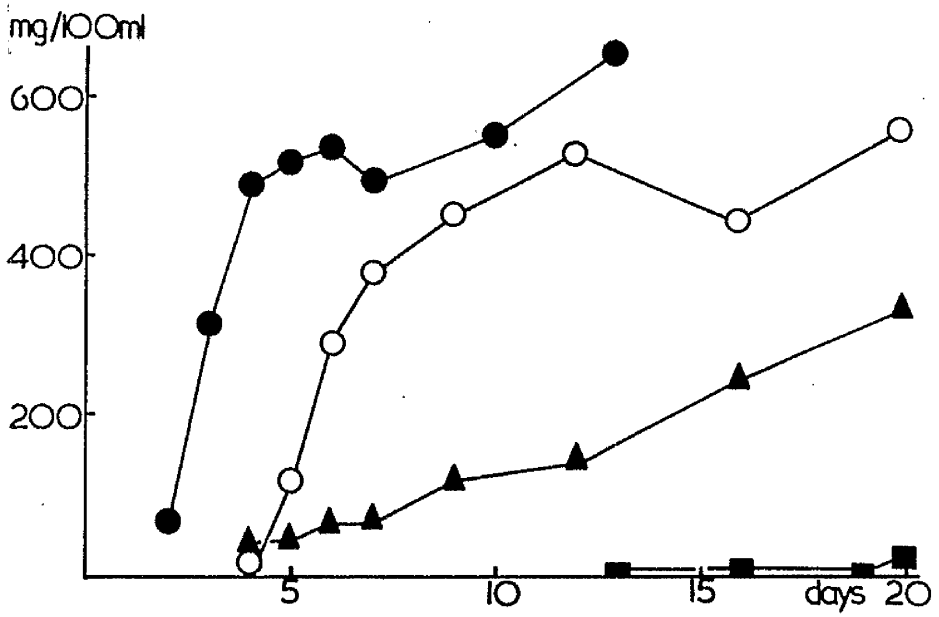


Fig.12 Effect of ethylenediaminetetraacetic acid (EDTA) on growth of stationary cultures of Aspergillus oryzae from large inocula. Control cultures from large (◉ - ◉) and small (○ - ○) inocula contained no EDTA. Other cultures from large inocula contained 250 mg/l (▲ - ▲) and 750 mg/l (■ - ■) EDTA.

Substrate: A_3 40g/l glucose

2323 Effect of ethylenediaminetetraacetic acid (EDTA)

Trace elements are able to participate in chelate complexes and have a recognized order of affinities, forming complexes of varying stability; EDTA has been widely used as a chelating or complexing agent in chemical and biochemical work. For instance Bartnicki-Garcia and Nickerson (1962) used EDTA in their study of Mucor rouxii and found that a yeast-like morphogenesis obtained under CO_2 was nullified by the action of EDTA, and normal filamentous growth obtained instead, showing that chelating agents do not necessarily confer toxicity. Agents such as EDTA offer a means of complexing unwanted trace elements in a substrate.

Published results (Moyeath, 1963) had shown that effect of size of inoculum was enhanced in substrates poor in trace elements. It was decided that an attempt should be made to free the substrate from trace elements further by a chelation process designed to block them. EDTA was chosen as chelating agent and Fig.12 shows its effect on cultures from large inocula. A concentration of 250 mg/l EDTA reduced growth rate and possibly maximum yield. A concentration of 750 mg/l EDTA suppressed growth almost

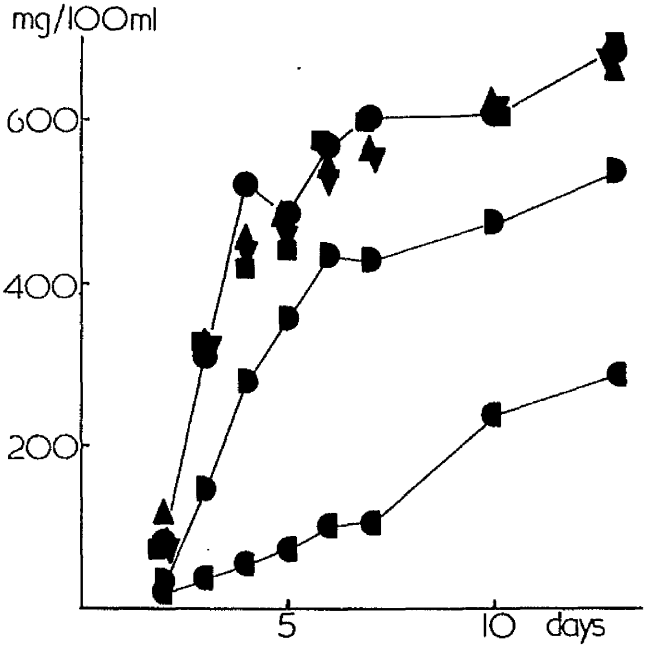


Fig.13 Effect of ethylenediaminetetraacetic acid (EDTA) on growth of stationary cultures of Aspergillus oryzae from large inocula.

	mg EDTA/l
◎ - ○	0
△ - △	1
□ - □	2.5
▽ - ▽	10
◗ - ◗	25
◖ - ◖	100

Substrate: A_3 40g/l glucose

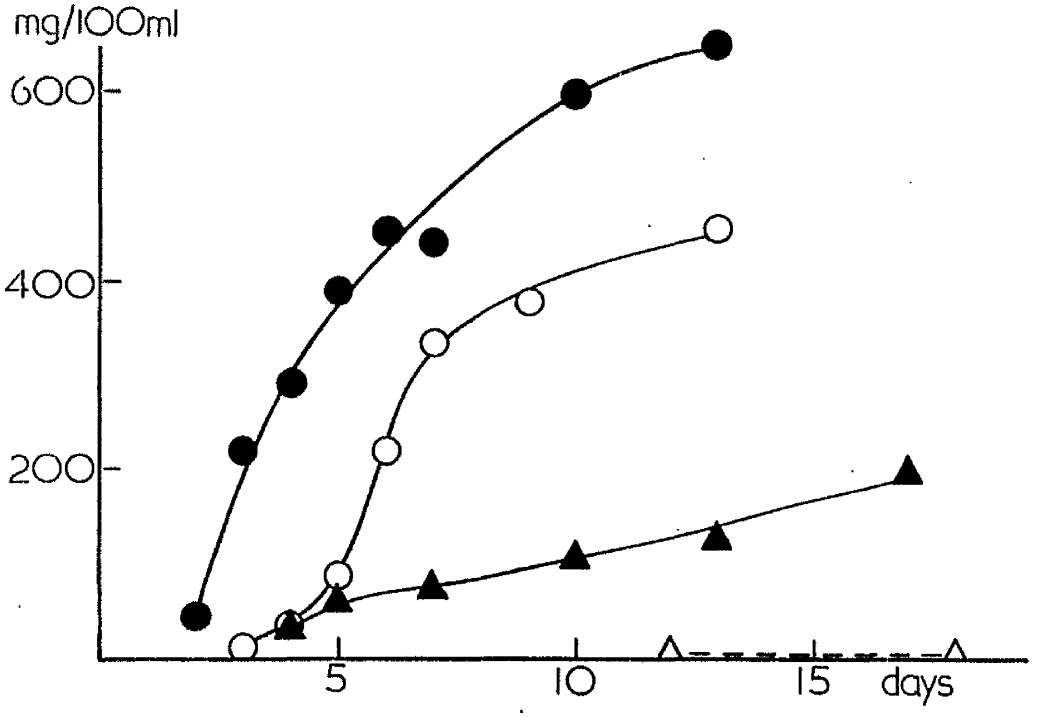


Fig.14 Effect of 250 mg/l ethylenediametetraacetic acid (EDTA) on growth of stationary cultures of EDTA from large and small inocula. Large-inoculum (● - ●) and small-inoculum (○ - ○) cultures were grown in absence of EDTA. Large-inoculum (▲- ▲) and small-inoculum (△-△) cultures were also grown in the presence of 250 mg/l EDTA.

Substrate: A_1 40g/l glucose

completely and so showed itself to be too high a concentration for use. A range of EDTA concentrations at a lower level was then tried for effect on large-inoculum cultures (Fig.13). Concentrations of up to 10 mg/l EDTA had little effect on growth rate and maximum yield, but concentrations of 25 and 100 mg/l increasingly depressed both of these features. When a concentration of 250 mg/l was tested against both large-inoculum and small-inoculum cultures, growth rate and maximum yield of the large-inoculum culture were reduced (Fig.14); growth of the small-inoculum culture was completely inhibited, showing that 250 mg/l is an unsuitable concentration for studies on effects of inoculum size if the physiological properties of the small-inoculum cultures are to be studied.

When a range of EDTA concentrations at a lower level was tried for effect on cultures from both large and small inocula, 10 mg/l had little influence on growth rate or maximum yield (Fig.15). A concentration of 25 mg/l EDTA reduced growth rate a little for both large-inoculum and small-inoculum cultures but reduced maximum yield more in the latter than in the former.

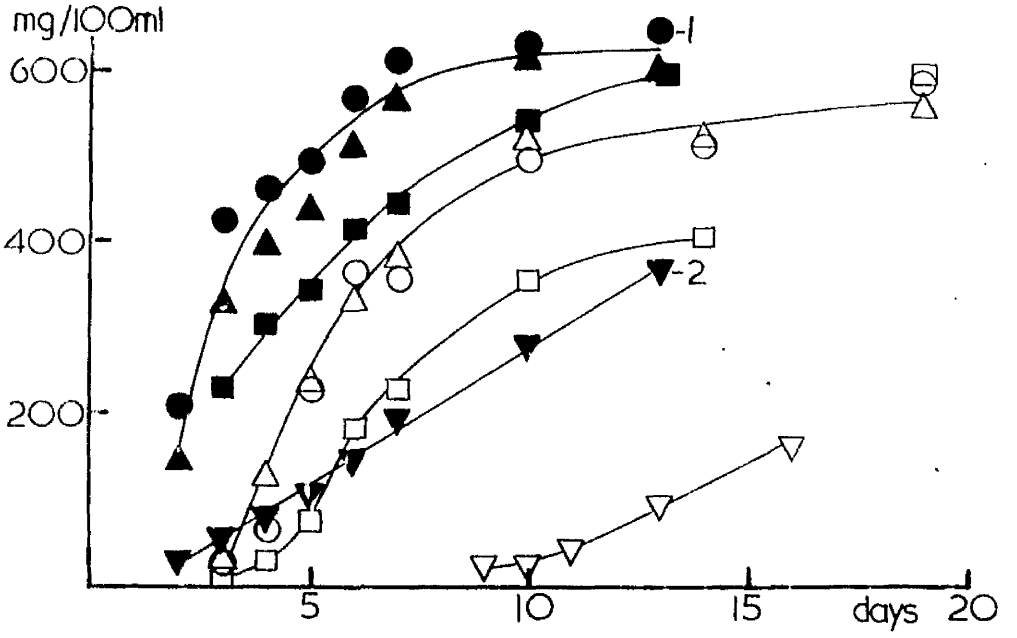


Fig.15 Effect of various concentrations of ethylenediaminetetraacetic acid (EDTA) on growth of stationary cultures of Aspergillus oryzae from large and small inocula. Filtrate was collected at sampling points 1 and 2 and used in a subsequent experiment (Fig.16).

large-inoculum cultures	small-inoculum cultures	mg/l EDTA
● - ●	○ - ○	0
▲ - ▲	△ - △	10
■ - ■	□ - □	25
▼ - ▼	▽ - ▽	100

Substrate: A_1 40g/l glucose

Table of Fig.15

Statistical analysis

	Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
●	samples 1-6	unacceptable	32.8	3.26		
○	samples 1-5	unacceptable	23.5	3.71		
▲	samples 1-6	unacceptable	7.15	3.26		
△	samples 1-5	unacceptable	10.1	3.71		
■	samples 1-5	52.3 ±2(4.49)	1.39	3.26		
□	samples 1-6	unacceptable	47.39	3.48		
▼	samples 1-8 (all samples)	31.72 ±2(0.85)	0.86	2.85		
▽	samples 1-5	22.4 ±2(2.01)	0.99	4.76		
	Comparison of these slopes				4.26	2.26
					difference significant	

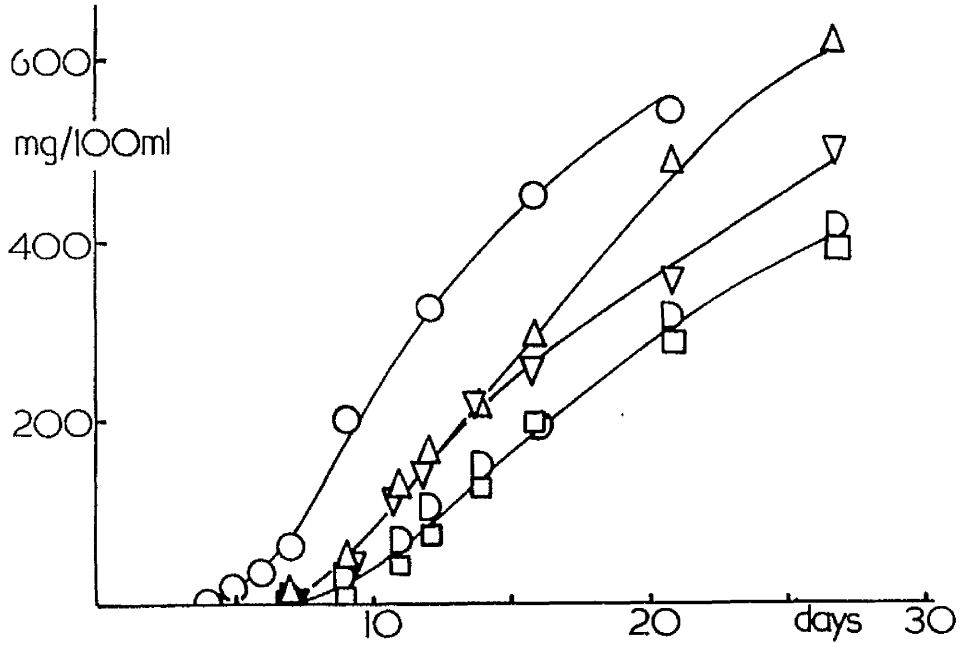


Fig.16 Effect of ethylenediaminetetraacetic acid (EDTA) and of culture filtrates on growth of stationary cultures of Aspergillus oryzae from small inocula.

	mg/l EDTA	Additive at inoculation
O - O	50	water
△ - △	75	water
□ - □	100	water
▽ - ▽	100	culture filtrate 1 (see Fig.15)
D - D	100	culture filtrate 2 (see Fig.15)

Culture filtrates 1 & 2 were collected from the sampling points so indicated in the preceding experiment (Fig.15).

Substrate: A_1 40g/l glucose

The result was that the effect of size of inoculum was more pronounced in respect of maximum yield. When the concentration of EDTA was increased to 100 mg/l, growth rate in both large-inoculum and small-inoculum cultures was reduced.

When statistical analysis was made of all or likely parts of the growth curves in Fig.15, in most cases a satisfactory straight line relationship could not be obtained (Table of Fig.15). This was no doubt due to a considerable extent to the curvature of the lines. Satisfactory straight line relationships were however obtained for both cultures containing 100 mg/l EDTA. When the two lines were compared, the difference in slope was found to be statistically significant (Table of Fig.15). Thus an effect of inoculum size has been demonstrated. Because of the nature of the curves and the delay in growth of the small-inoculum culture, little inference could be drawn in respect of maximum yields.

In a further experiment, using small-inoculum cultures only, a progressive increase in EDTA concentration (50 to 75 to 100 mg/l) gave a progressive decrease in rate of growth (Fig.16). The effects of addition of culture filtrate shown in Fig.16 are dealt with later (p. 101).

The results from EDTA experiments had thus shown that 250 mg/l EDTA inhibited growth of small-inoculum cultures whereas a concentration as high as 750 mg/l was required to inhibit growth of large-inoculum cultures. An effect of size of inoculum was shown for certain EDTA concentrations below 250 mg/l. Thus a range had been established in which EDTA was operative in the direction that had been sought.

2324 Effect of purification of glucose

Thus far unavailability of trace elements for the growth of the mould has been considered on the basis of their not being added to the substrate or their being blocked by a chelating agent. Other methods remain. Biological depletion was practised almost a century ago by Raulin (cited by Nicholas 1963a) who reduced the Fe and Zn content of a culture solution by growing in it several generations of *A. niger*. MacLeod and Snell (1950) used this method more recently but Nicholas (1963a) considers it "unsatisfactory since several other constituents of the media are also depleted during growth".

Such a procedure however does not only deprive a substrate of factors needed for growth. It contributes an unknown number of factors such as products of the excretory metabolism of the organism used in depletion. Such products may affect growth of the test culture after removal of the organism used for depletion.

Purification techniques were reviewed by Foster (1949). Organic compounds, in particular hydroxy and polyhydroxy compounds and amino acids, typically form complexes with trace elements and cationic impurities (see Foster, 1949). Glucose was thus a likely source of trace element contamination in the tests reported below, hence it was decided to purify a solution of the glucose before its incorporation into the final substrate. Active carbon has been used for such purification as have co-precipitation methods involving calcium carbonate and copper sulphide (see Nicholas 1963a). An aluminium hydroxide co-precipitation method was selected for use here and is described above (p. 36). Accordingly, before glucose was added to the remaining constituents of the substrate, it was purified in this way. The influence of this treatment on the effect of size of inoculum was then obtained by a study of the growth curves with reference particularly to growth rate and maximum yield.

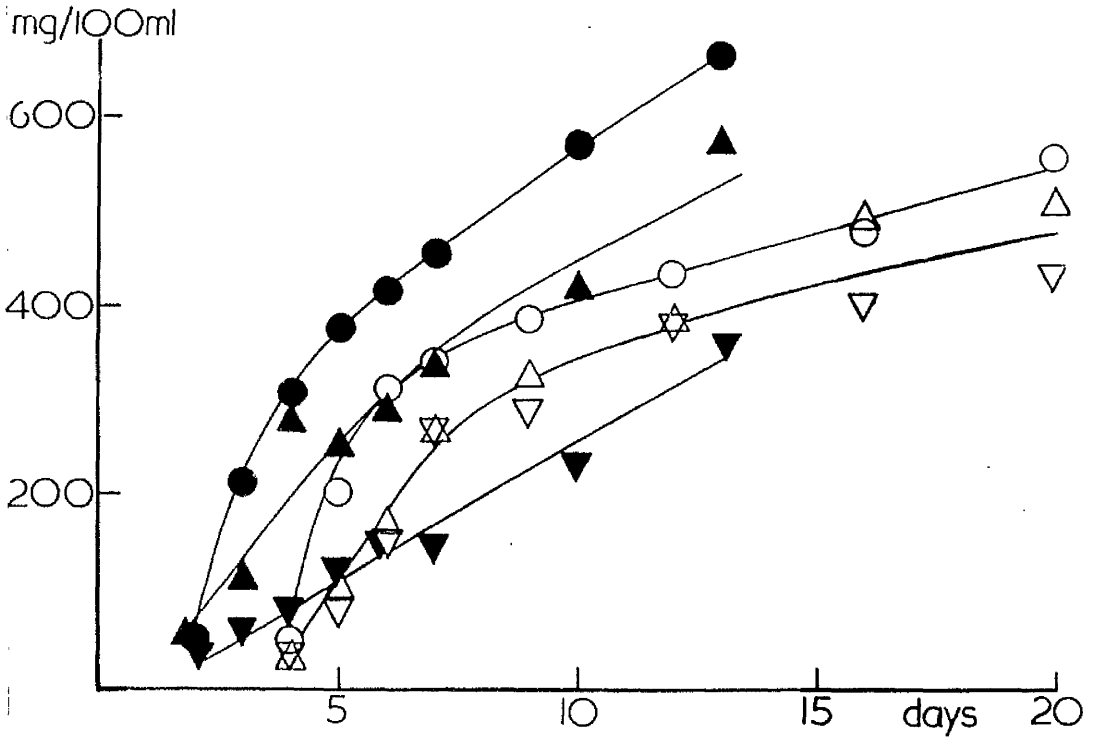


Fig.17 Effect of purification of the glucose in the substrate by aluminium hydroxide treatment at pH values of 5.5 and 8.5 on the growth of stationary cultures of Aspergillus oryzae from large and small inocula.

large-inoculum cultures	small-inoculum cultures	aluminium hydroxide treatment
● - ◉	○ - ○	nil
▲ - ▲	△ - △	at pH value of 5.5
▼ - ▽	▽ - ▽	at pH value of 8.5

Substrate: A_1 40g/l glucose

Table of Fig.17

Statistical analysis

Range	Regression coefficient	'r' actual	'r' theoretical	't' actual	't' theoretical
● , samples 1-3	*126.67 ±2(16.66)	6.07	5.99		
○ , samples 1-3	131 ±2(12.12)	3.61	5.99		
Comparison of these slopes				0.19	4.30 difference not sig.
● , samples 3-8 (last six)	38.83 ±2(2.1)	0.99	3.26		
○ , samples 3-8 (last six)	16.81 ±2(0.96)	0.30	3.26		
Comparison of these slopes				9.53	5.04 (0.1%) difference v. highly sig.
▲ , samples 1-6	55.45 ±2(11.81)	1.89	3.26		
△ , samples 1-5	unacceptable	5.33	3.71		
▼ , samples 1-4	28.13 ±2(3.04)	0.34	4.46		
▽ , samples 1-4	78.67 ±2(10.11)	1.72	4.46		
Comparison of these slopes				4.79	4.60 (1%) difference highly sig.

* The regression coefficient of the line for these three samples (● - ●) is cited and used in comparison of the slopes although the 'F' value is just outside the desired limit; see text.

Fig.17 shows the results obtained from cultures in substrates containing glucose purified at pH values of 5.5 and 8.5 in turn. In comparing cultures from different sizes of inoculum in which the glucose had been untreated (● - ● and O - O) there seemed to be little difference in initial rate of growth between the two cultures, but a difference in rate at late stages. These inferences were confirmed statistically (Table of Fig.17). The first three samples for the small-inoculum culture (O - O) could be represented by a straight line. The regression line for the corresponding parts of the large-inoculum curve (● - ●) fell just outside the limits of acceptance as a straight line. As it was on the borderline of acceptance, it was taken as a straight line for the purpose of comparison with the line from the small-inoculum culture. As can be seen, the slopes of the lines do not in any case differ significantly, nor is their relationship near to significance. It may be assumed, then, that there is no effect on rate of growth at early stages due to differences in inoculum size. At late stages, however, an effect of inoculum size was confirmed. The last six points of each culture (● - ● and O - O) could be represented by a straight line in each case. The

difference between the slopes of these lines was very highly significant (Table of Fig.17). An effect of inoculum size is therefore evident at the later stages of growth.

The growth curves given by cultures grown from glucose purified at a pH value of 5.5 (▲-▲and△-△) did not give an obvious effect due to size of inoculum. Statistically, moreover, although the first six samples of the large-inoculum culture (▲-▲) could be represented by a straight line, the first five of the small-inoculum culture (△-△) could not.

When glucose was purified at a pH value of 8.5, a 'reversal' phenomenon became evident; the large-inoculum culture gave a lower growth rate than the small-inoculum culture, a reversal of what has generally been true when an inoculum-size effect has been shown. This reversal is confirmed by statistical analysis; the first four points of each curve (▼-▼and▽-▽) can be represented by straight lines, and the divergence between those lines is highly significant (Table of Fig.17). Therefore both a 'normal' effect (using untreated glucose) and a 'reversal' effect (using glucose treated at a pH value of 8.5) is shown in Fig.17. This reversal was also found when the purification was performed at a pH value of 7, as can be seen from the controls of several subsequent experiments (e.g.Fig.18).

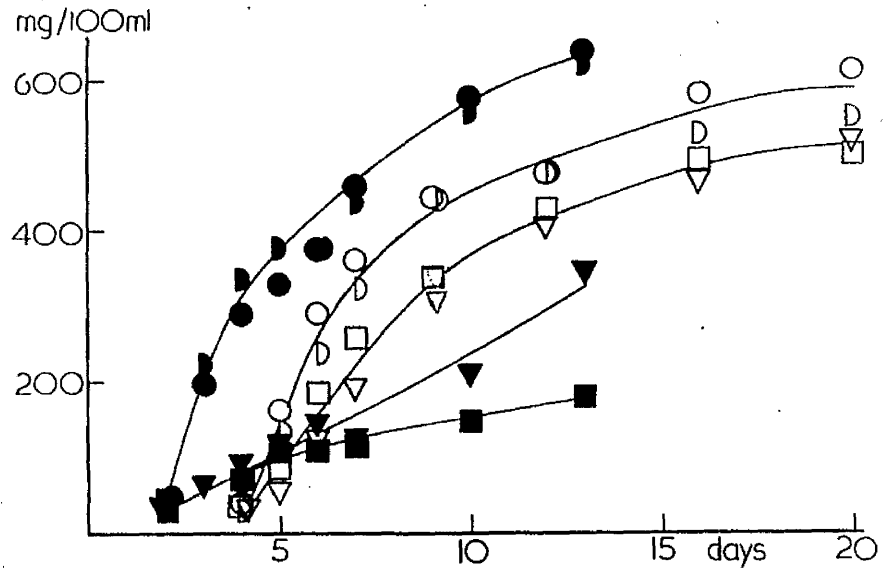


Fig.18 Effect of trace-element supplementation of glucose-purified (pH 7) substrates on the growth of stationary cultures of Aspergillus oryzae from large and small inocula.

	large-inoculum cultures	small-inoculum cultures	treatment
1	⊙ - ⊙	○ - ○	unpurified glucose
2	⊡ - ⊡	□ - □	purified glucose
3	▽ - ▽	▽ - ▽	purified glucose + Fe Ca Mn Cu
4	D - D	D - D	purified glucose + Fe Ca Mn Cu Zn

Substrate: For 1 & 2, A_1 40g/l glucose; for 3, A 40g/l glucose except that it has twice (30 mg/l) the $FeCl_3.6H_2O$ and no Zn; for 4, A 40g/l glucose except that it has twice (30 mg/l) the $FeCl_3.6H_2O$ and 1/10th (0.5 mg/l) the $ZnSO_4.7H_2O$.

Table of Fig.18

Statistical analysis

Range	Regression coefficient		'F' actual	'F' theoretical	't' actual	't' theoretical
■ , samples 1-7 (all samples)	12.96	±2(1.52)	1.36	3.20		
□ , samples 1-5	63.9	±2(5.77)	2.05	3.71		
Comparison of these slopes					8.54	3.04 (0.1%) difference very highly significant
▼ , samples 1-8 (all samples)	25.87	±2(2.44)	0.73	2.74		
▽ , samples 1-6	90	±2(2.17)	0.99	3.26		
Comparison of these slopes					2.77	2.23 difference significant

Fig.18 shows the results of an experiment devised to confirm that the reversal was due to the removal of trace elements from the substrate. A substrate containing unpurified glucose gave rise to growth curves in which the large-inoculum culture was, as usual, always ahead of the small-inoculum culture in that at any given point of time the sample from the large-inoculum culture was of a greater weight. The use of glucose purified at a pH value of 7 gave a marked reversal of these results. This reversal was statistically confirmed. The whole of the curve for the large-inoculum culture (■-■) could be represented by a straight line, as could the first five points of the curve for the small-inoculum culture (□-□). When compared, the difference between these lines was found to be very highly significant (Table of Fig.18).

Furthermore, the addition of a combination of Fe, Ca, Mn and Cu counteracted the reversal in part. The reversal was still significant as is shown by the Table of Fig.18 in which the slope of the straight line derived from the first six points of the small-inoculum culture (▽-▽) is compared with the line representing all the points of the large-inoculum culture.

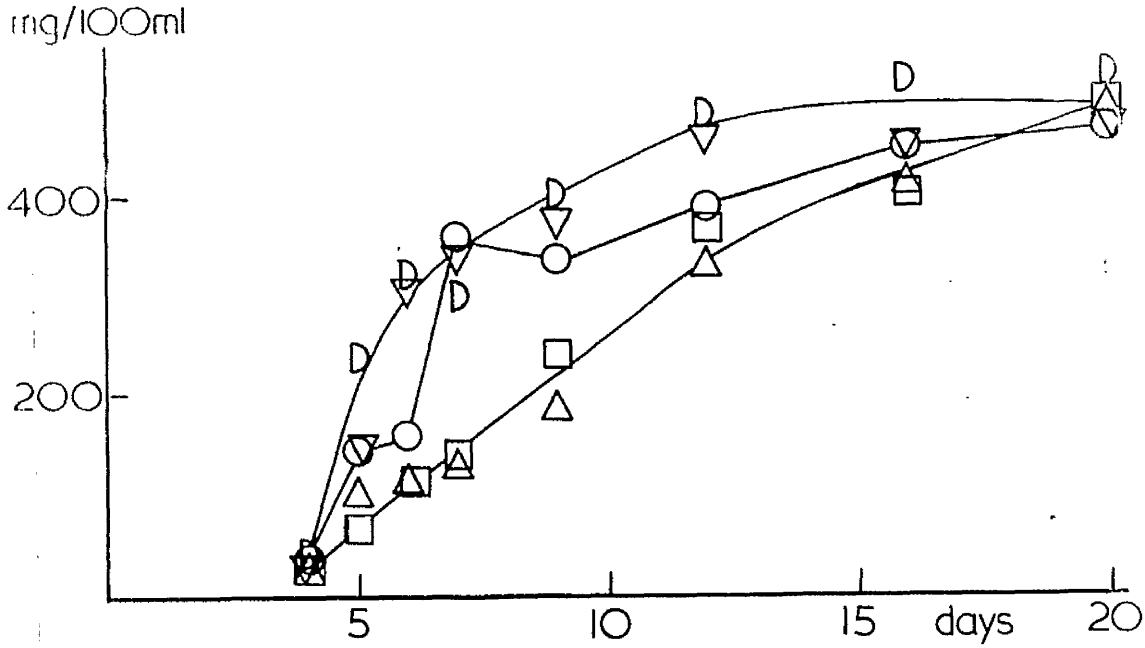


Fig.19 Effect of graded concentrations of Zn on growth of stationary cultures of Aspergillus oryzae from small inocula in a glucose-purified (pH7) substrate with Fe, Ca, Mn and Cu as trace elements. The concentrations of Zn were (as $ZnSO_4 \cdot 7H_2O$ mg/l):
o - o, none; △ - △, 0.005; □ - □, 0.05;
▽ - ▽, 0.5; D - D, 5.0.

Substrate: A 40g/l glucose but with twice $FeCl_3 \cdot 6H_2O$ (30 mg/l) and variable $ZnSO_4 \cdot 7H_2O$.

Table of Fig.19

Statistical analysis

Days	Sample No.	Comparison of ○ and △ 't'	significance	Comparison of ○ and □ 't'	significance
4	1	1.14	not sig.	0.72	not sig.
5	2	0.95	not sig.	7.41	highly sig.
6	3	1.38	not sig.	2.00	not sig.
7	4	14.77	v.highly sig.	26.08	v.highly sig.
9	5	3.73	significant	2.21	not sig.
12	6	1.36	not sig.	1.67	not sig.
16	7	3.62	significant	3.10	significant
20	8	0.72	not sig.	1.27	not sig.

Theoretical values of 't' throughout:

5% (significant) 2.78

1% (highly sig.) 4.60

0.1% (v.highly sig.) 8.61

continued on next page...

Table of Fig.19 ... continued from previous page

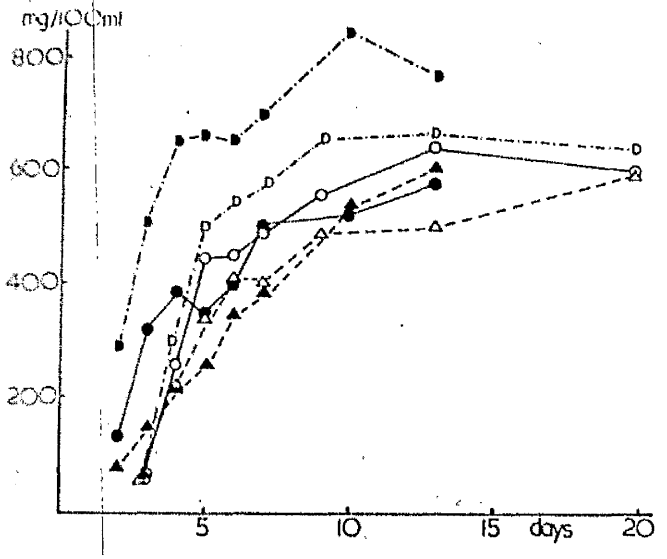
Statistical analysis

		Comparison of ○ and ▯		Comparison of ○ and ▽	
Days	Sample No.	't'	significance	't'	significance
4	1	0.43	not sig.	0.99	not sig.
5	2	8.43	highly sig.	0.19	not sig.
6	3	7.15	highly sig.	6.02	highly sig.
7	4	6.00	highly sig.	2.09	not sig.
9	5	1.60	not sig.	0.85	not sig.
12	6	5.49	highly sig.	5.44	highly sig.
16	7	4.28	significant	0.43	not sig.
20	8	2.42	not sig.	0.23	not sig.

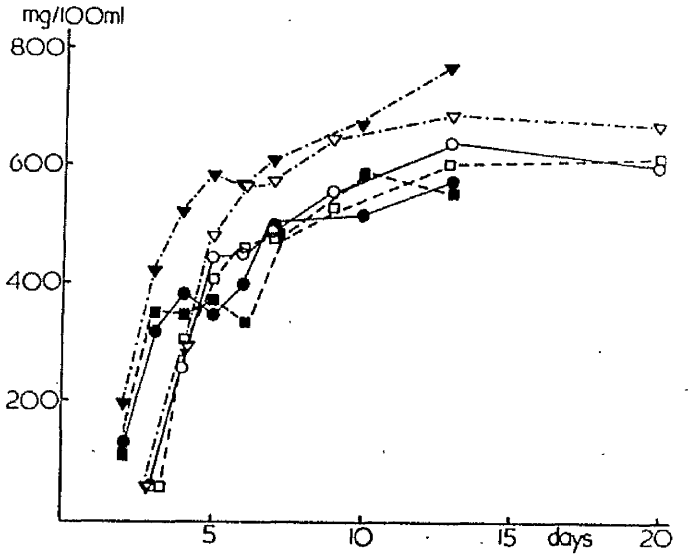
		Comparison of △ and ▯	
Days	Sample No.	't'	significance
4	1	3.88	significant
5	2	3.09	significant
6	3	7.52	highly sig.
7	4	10.42	v.highly sig.
9	5	20.15	v.highly sig.
12	6	3.51	significant
16	7	6.26	highly sig.
20	8	1.66	not sig.

It is interesting to note that the addition of these same trace elements (Fe, Ca, Mn and Cu) together with Zn completely counteracted the reversal to give results almost indistinguishable from those for the culture containing unpurified glucose (Table of Fig.18).

The role of Zn in size-of-inoculum phenomena was investigated further. To substrates containing glucose purified at a pH value of 7, Fe, Ca, Mn and Cu were added as trace elements together with graded concentrations of Zn. The results are recorded in Fig.19 and the statistical examination of those in the Table of Fig.19. Due to the curvature of the lines, statistical analysis was performed by 't' tests between the points at each stage. As the graph indicates and the statistical analysis confirms, there is (with one exception) no significant difference between any of the variants at the first and last points of sampling. At the intermediate points of sampling, the divergence of results from the control varied from non-significant to very highly significant. It should be noted that when the two smallest amounts of Zn were added ($\triangle - \triangle$ and $\square - \square$), on three out of six occasions in each of the two cases growth weights significantly smaller than those for



(a)



(b)

Fig. 20 Effect of graded concentrations of Zn on growth of stationary cultures of Aspergillus oryzae from large and small inocula in a glucose-purified (pH7) substrate with Fe, Ca, Mn and Cu as trace elements.

	large-inoculum cultures	small-inoculum cultures	mg/l $ZnSO_4 \cdot 7H_2O$
a)	▲ - ▲	△ - △	0.005
a)	◗ - ◗	D - D	5.0
b)	● - ●	○ - ○	none
b)	■ - ■	□ - □	0.05
b)	▼ - ▼	▽ - ▽	0.5

Substrate: A 40g/l glucose but with twice the $FeCl_3 \cdot 6H_2O$ (30 mg/l) and variable $ZnSO_4 \cdot 7H_2O$

Table of Fig.20

Statistical analysis

	Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
D	samples 1-6	unacceptable	5.09	3.26		
▲	samples 1-6	60.97 ±2(3.01)	0.11	3.26		

	Samples compared	't' actual	't' theoretical	significance
D	10 day (penultimate sample) and 13 day (penultimate sample) D	6.16	4.60 (1%)	difference highly significant

similar cultures with no Zn added (O - O) were recorded. Similarly when the largest amounts of Zn were added (▽- ▽ and D - D), on a total of seven out of twelve occasions, growth weights significantly larger than those for similar cultures with no Zn added (O - O) were recorded. When in turn the series containing the smallest amount of Zn (△- △) was compared with the series containing the largest amount of Zn (D- D) the former gave rise to growth weights always significantly smaller than the latter. The general indication is that small amounts inhibit, larger amounts stimulate growth.

These effects of Zn were examined in greater detail (Fig.20 a and b) with both large and small inocula. It should be noticed in passing that the control cultured showed the reversal effect on using a substrate containing glucose purified at a pH value of 7. The curved nature of the lines made them unlikely material for reduction to statistically acceptable straight lines. For example, in Fig.20 a, with large-inoculum cultures, an acceptable straight line could be found for the first six samples of the culture with the small amount of Zn (▲-▲), but not for the first six samples of the culture with the large amount of Zn (● - ●, Table of Fig.20). For both large-

and small-inoculum cultures, however, the tendency was present throughout (sometimes shown very markedly) for a small amount of Zn (0.005 mg/l $ZnSO_4 \cdot 7H_2O$) to be inhibitory as compared with no Zn while a large amount of Zn (5 mg/l $ZnSO_4 \cdot 7H_2O$) was stimulatory. At the point of greatest yield of the cultures containing most Zn (5 mg/l $ZnSO_4 \cdot 7H_2O$) there was a highly significant difference in yield between large-inoculum and small-inoculum cultures (Table of Fig.20).

The same pattern is repeated with two other concentrations of Zn (Fig.20b), and the role of Zn is undoubtedly a complex one. Commonly a small amount of a substance may be stimulatory and a larger amount inhibitory. In these figures (Figs.19, 20a and 20b) the reverse is true; small amounts are inhibitory and larger amounts stimulatory.

The role of Fe in inoculum-size phenomena was also examined in further detail. Although the effect of addition of Fe had been examined, the addition had been to a basal substrate already containing Fe. The result of tests in which Fe was omitted in its entirety from the basal substrate are shown in Fig.21. It can be seen that when Fe was completely omitted from the substrate, there

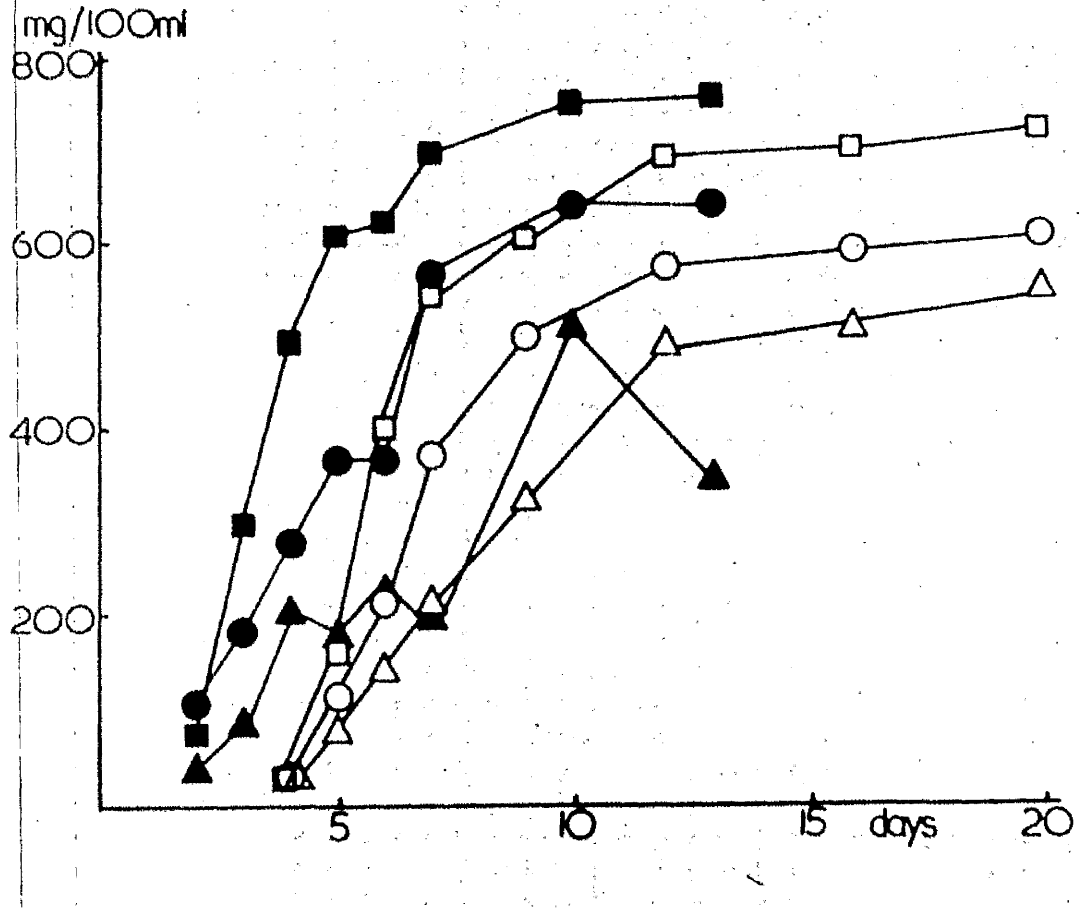


Fig.21 Effect of Fe on growth of stationary cultures of Aspergillus oryzae from large and small inocula in glucose-purified (pH7) substrates.

	large-inoculum cultures	small-inoculum cultures	trace-element addition
1	● - ●	○ - ○	none - (and no Fe in basal substrate)
2	▲ - ▲	△ - △	Fe only
3	■ - ■	□ - □	Fe, Ca, Mn, Cu, Zn

Substrate: For 1, A_1 40 g/l glucose but with no Fe.
For 2, A_1 40 g/l glucose
For 3, A 40 g/l glucose but with 1/10th (0.5 mg/l) the $ZnSO_4 \cdot 7H_2O$

Table of Fig.21

Statistical analysis

Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
○ , samples 1-6	unacceptable	8.32	3.26		
○ , samples 1-5	97.6 ±2(8.25)	1.64	3.71		
● , samples 1-6	83.75 ±2(10.12)	1.40	3.26		
Comparison of these slopes				1.08	2.36 difference not significant
▲ , samples 1-6	33.55 ±2(10.56)	1.66	3.26		
△ , samples 1-6	58.6 ±2(0.42)	0.08	3.26		
Comparison of these slopes				2.35	2.31 difference significant
■ , samples 1-4	180 ±2(17.62)	1.45	4.46		
□ , samples 1-4	178.6 ±2(14.8)	4.07	4.46		
Comparison of these slopes				0.06	2.78 difference not significant

was no reversal effect such as usually occurred in a substrate containing both glucose purified at a pH value of 7 and Fe (see Fig.18, purified glucose). Statistical examination was made of the curves of Fig.21 (see Table of Fig.21). Acceptable straight lines could be obtained to represent the first six samples of the large-inoculum culture with no Fe whatsoever (● - ●) and the first five samples (but not the first six) of the corresponding small-inoculum culture (○ - ○). The difference between the slopes of these lines was not significant and moreover, the commonly found relationship between cultures from large and small inocula held throughout, namely, that the former had a mycelial content greater than the latter at any one time. When Fe was now added, the 'reversal' effect occurred. This was shown by comparison of the slopes of the straight lines representing the first six samples of each culture (▲- ▲ and △ -△). The difference between these lines was statistically significant (Table of Fig.21), the lower growth rate being that of the large-inoculum culture. Addition of a combination of Fe, Ca, Mn, Cu and Zn countered this reversal completely. This was shown by the remaining curves (■ - ■ and □ - □). The first four samples of each curve gave acceptable straight lines (see

table of Fig.21) and these lines did not differ significantly in slope; that is, the rates of growth of the cultures did not differ significantly.

Collating the information of these two figures (Figs.20 and 21), it may be said that without addition of Fe or of trace elements, purification of the glucose at a pH value of 7 had no influence on the effect of size of inoculum (Fig.21, ● and ○): addition of Fe only gave the reversal effect (Figs.18, ■ and □ ; 21, ▲ and △): addition of Co, Mn and Cu as well as Fe countered the reversal effect in part (Fig.18, ▼ and ▽): addition of all four of these and Zn in addition countered the reversal effect completely (Figs.18, ◗ and ◫ ; 21, ■ and □).

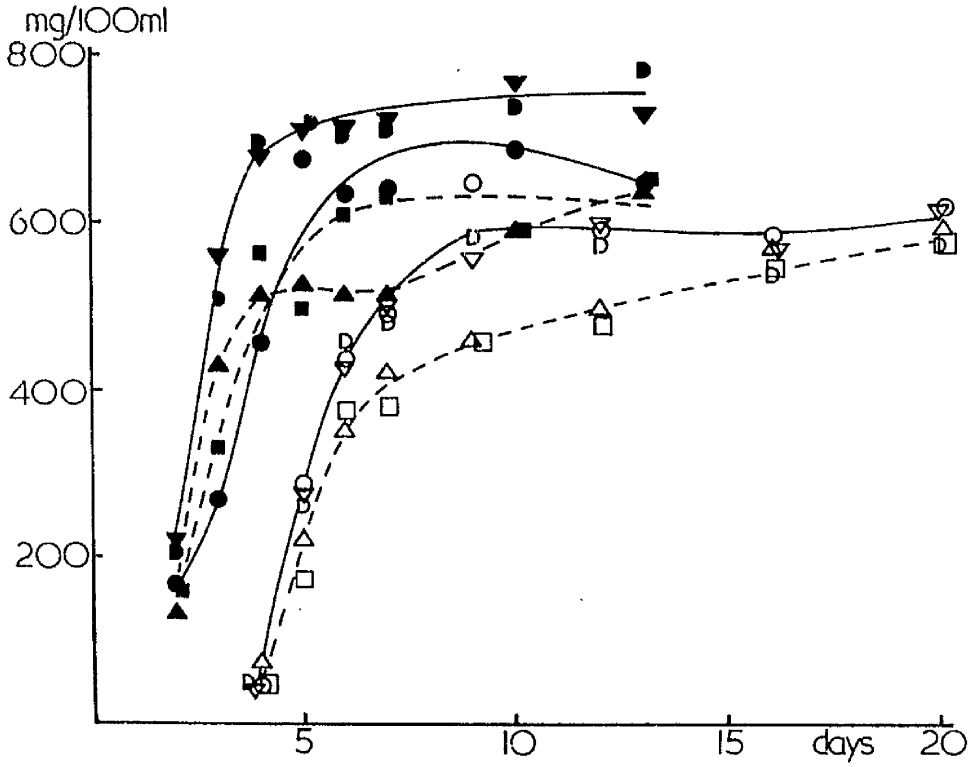


Fig.22 Effect of caramelization on growth of stationary cultures of Aspergillus oryzae from large and small inocula when graded concentrations of Zn were used in a glucose-purified (pH7) substrate with Fe, Ca, Mn and Cu as trace elements.

large-inoculum cultures	small-inoculum cultures	$ZnSO_4 \cdot 7H_2O$ mg/l
● - ●	○ - ○	0
▲ - ▲	△ - △	0.005
■ - ■	□ - □	0.05
▼ - ▼	▽ - ▽	0.5
◗ - ◗	D - D	5

Substrate: A 40g/l glucose but with twice $FeCl_3 \cdot 6H_2O$ (30 mg/l), variable $ZnSO_4 \cdot 7H_2O$ and caramelization throughout.

Table of Fig.22

Statistical analysis

Samples compared	't' actual	't' theoretical	significance
● and ▼; 10 day (penultimate samples)	1.02	2.78	not sig.
● and ◗; 10 day (penultimate samples)	0.84	2.78	not sig.
● and ▲; 10 day (penultimate samples)	1.93	2.78	not sig.
● and ■; 10 day (penultimate samples)	1.30	2.78	not sig.
◗ and ●; 13 day (last samples)	5.36	4.60 (1%)	highly sig.
◗ and ▲; 13 day (last samples)	3.20	2.78	significant
◗ and ■; 13 day (last samples)	3.98	2.78	significant

Range	Regression coefficient	'F' actual	'F' theoretical	't' actual	't' theoretical
▲, samples 1 - 3	190 $\pm 2(61.9)$	1.13	5.99		
△, samples 1 - 3	137.83 $\pm 2(3.89)$	0.21	5.99		
Comparison of these slopes				0.84	4.30 difference not significant

2325 Effect of caramelization

In one series of experiments, it was noted that the substrate had become caramelized during preparation, and it was already known that caramelization could affect size-of-inoculum phenomena (Meyrath, 1963). The results obtained when the caramelized substrate was used are shown in Fig.22. The experiments recorded in Fig.20 are identical to those recorded in Fig.22 in every respect except that the substrates in the latter case showed caramelization. It will be noted that the effect seen in Fig.20 in which cultures from large and small inocula were inhibited by small concentrations of Zn and stimulated by large, is repeated here. The inhibitory effect showed most strongly with the small-inoculum cultures and the stimulatory effect with the large-inoculum cultures.

The penultimate samples for the large-inoculum culture were examined statistically (Table of Fig.22) but no significant difference was found between the mycelial content of the control cultures (no Zn) and that of cultures containing any other concentration of Zn (Table of Fig.22). The last samples of the large-

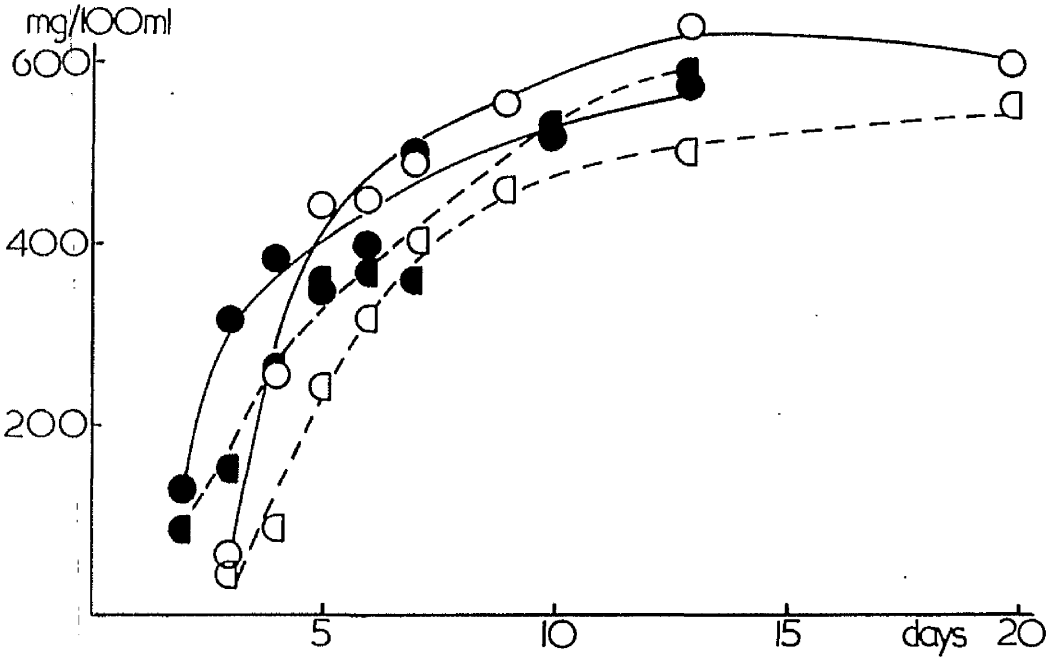


Fig.23 Effect of deliberate caramelization on the growth of stationary cultures of Aspergillus oryzae from large and small inocula in a glucose-purified (pH7) substrate with Fe, Ca, Mn and Cu as trace elements. Large-inoculum (● - ●) and small-inoculum (○ - ○) control cultures (continuous lines) are as for Fig.20. Similar large-inoculum (◪ - ◪) and small-inoculum (□ - □) cultures were treated to give caramelization (broken lines).

Substrate: A 40g/l glucose but with twice $FeCl_3.6H_2O$ (30 mg/l)

inoculum culture did, however, show significance in difference of mycelium content between the culture containing most Zn (◗ - ◗) and those cultures containing no Zn (● - ●) or the two smallest amounts of Zn (▲-▲and■-■), as is shown in the Table of Fig.22. Again, the indication is of stimulation by large amounts of Zn. The stimulatory and inhibitory role of Zn is discussed later.

There was little apparent difference in rate of growth of cultures due to the size of the inoculum. This was confirmed statistically in one case. No significant difference existed in the slope of the lines representing the first three samples of the cultures with the least amount of Zn (▲-▲and△-△), as is shown in the Table of Fig.22. The reversal effect was thus not observed on this occasion, and caramelization was held responsible.

Corroboration of the suggestion that caramelisation was responsible for loss of the reversal phenomenon is given in Fig.23. In this experiment caramelisation was effected deliberately by the procedure described in section 226. In Fig.23, the control cultures of Fig.20 are compared with similar cultures which had been deliberately caramelised. The reversal phenomenon shown in the uncaramelised substrate was completely countered on caramelisation.

233 Self-stimulatory and self-inhibitory substances

There must be very few substances known which do not, in some concentration, stimulate or inhibit growth of a culture in an observable way, and a wide range of literature exists on such action. Here, attention is focussed on instances where the stimulatory or inhibitory substance is a product of an organism's own metabolism, i.e. where the substance is to be found in the filtrate of the culture. In particular, a correlation between the production of such substances and the effects of inoculum size has been sought.

Walker, Reeves and Lankford (1962) made a contribution to the literature which has special reference to inoculum size. They studied the kinetics of production of endogenous cell division activators in relation to inoculum density in cultures of Bacillus megaterium. Activators of cell division were excreted into the medium and were shown to be present in the filtrates. It is interesting to note that there was no direct proportionality between number of cells present and amount of activator. Instead, there was more activator per cell produced from a small inoculum, so that the lag of a small-inoculum culture was shorter than

would be expected. Heyrath (1963) showed that with Aspergillus oryzae, a small-inoculum culture did not give a markedly increased lag time over large-inoculum cultures, possibly due to a mechanism similar to that described by Walker, Reeves and Lankford.

Harwood (1956) described a substance produced by cultures of Bronycos phaseoli which was self-stimulatory with respect to the length of germ tubes of spores. McKenzie, Hutchinson and Reed (1959) described a volatile substance stimulating spore germination in Agaricus campestris which was identified as 2:3-dimethyl-1-pentene, and Hepden & Hawker (1961) a volatile substance which controlled zygospore formation in Rhizopus sexualis. Darling and McArdle (1959) were able to 'prime' or modify media with substances produced by a large inoculum of a mutant strain of Aspergillus amstelodami in such a way that the medium supported growth of single-spore cultures, although it had not been able to do so previously. Padwick (1939) explained an increase in germination of large inocula of ascospores of Ophiobolus graminis on the basis of excretion of stimulating substances by the ascospores or perithecia.

Not all self-produced substances are stimulatory.

So-called 'staling' has been a long-recognised property of certain fungal cultures; this is evidenced by a loss of circular outline to the colony on solid media whereas in liquid cultures it is demonstrated by a fall in rate of growth (Hawker, 1950). It is not a starvation phenomenon, but is a result of self-produced inhibitors (Hawker, 1950). Cochrane (1958) suggests that in substrates containing a high concentration of carbohydrate the staling substances are probably organic acids and that in substrates having a high content of nitrogen, the staling substance is ammonia.

Several other studies of self-produced inhibitors are to be found in the literature, some of these being gaseous. Yarwood (1956) and Allen (1955) found such self-inhibitors involved in the germination of urediospores of Uromyces phaseoli and Puccinia graminis f.sp tritici respectively. Allen (1955) considered that neither reduced oxygen tension nor increased carbon dioxide tension was responsible for the effect he described. Allen showed that on testing 3×10^3 spores, the percentage germination of conidia increased almost linearly with time, but on testing 3×10^5 spores, germination was almost totally inhibited. Carlile and Gellin (1963) also showed

that a factor produced by mycelium of Botrytis cinerea was inhibitory towards the germination of its own spores and Schopfer (1933) reported a similarly acting substance in Phycomyces blakesleeanus.

Meyrath (1962a) has shown that small amounts of filtrate from cultures of Aspergillus oryzae of varying age influence both rate of growth and maximum yield of the culture when added at the time of inoculation. It has also been shown that the influence of such filtrates on growth rate is not so pronounced when the mycelium of the inoculum is taken from older cultures (Meyrath, 1962a). From these findings it was concluded that growth-promoting substances produced at early and at later stages of growth of a culture affected the behaviour of such a culture at much later stages provided these substances acted at a sufficiently early stage. Moreover, it was shown that synthesising activity of the mycelium in a culture decreased at first, then increased slowly. This was thought to be due to the excretion of growth-inhibiting substances at an even earlier stage than the growth-stimulating substances. The section which follows describes how stimulatory and inhibitory substances were sought in filtrates of both stationary and submerged cultures from both large and small inocula.

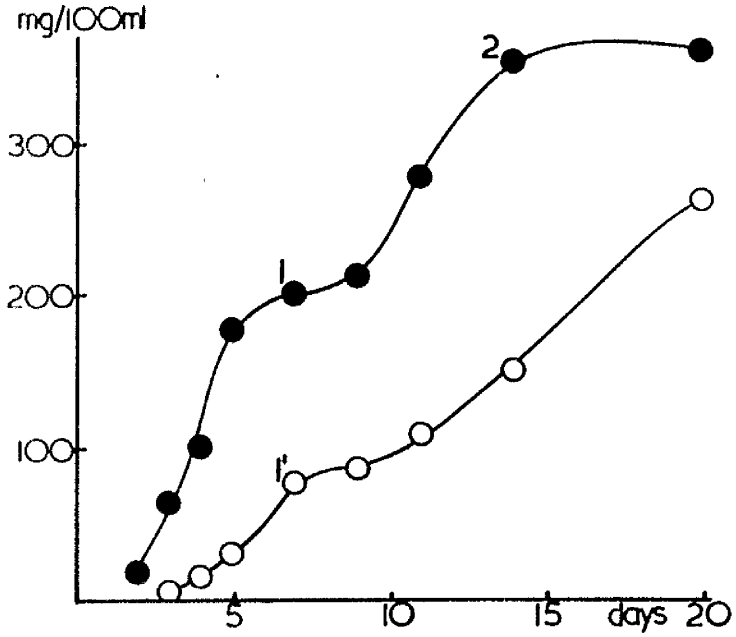


Fig.24 Growth pattern of cultures of Aspergillus oryzae grown in stationary conditions from large (◎ - ◎) and small (O - O) inocula. Filtrates were collected at points 1 and 2 (large-inoculum culture) and at point 1' (small-inoculum culture) and used for testing (courtesy of J.Meyrath).

Substrate: A_3 40g/l glucose except that $FeCl_3.6H_2O$ was 3 mg/l

Table of Fig.24

Statistical analysis

	Range	Regression coefficient	'r' actual	'r' theoretical	't' actual	't' theoretical
●	♀ samples 1-8 (all samples except last)	25.99 ±2(2.48)	not obtainable (single readings only available throughout)			
○	♀ samples 1-8 (all samples)	14.6 ±2(0.73)				
	Comparison of these slopes				4.41 very highly significant	4.32 (0.1%)

2331 Self-stimulatory and self-inhibitory substances in stationary cultures

Filtrates from stationary cultures are considered first. The growth curves for the cultures from which the filtrates were taken are shown in Fig.24. The difference between the slopes of the two regression lines representing eight samples of each curve (Table of Fig.24) was very highly significant, showing an effect of size of inoculum even under stationary conditions. At points 1 and 2 (large-inoculum culture) and at 1' (small-inoculum culture) the filtrates were retained when the mycelium was being collected for weight estimation, and these filtrates added to fresh cultures in the manner described earlier (pp.35,40). Steamed substrate was used as a control because the added filtrate had been steamed to sterilise it (30 min. on 3 successive days). Although the substrate had been heat-sterilised (by autoclaving), the usual practice had been followed of sterilizing the glucose separately from the minerals. Steaming the filtrate might then have given rise to caramelization and this steamed substrate control allowed for that possibility.

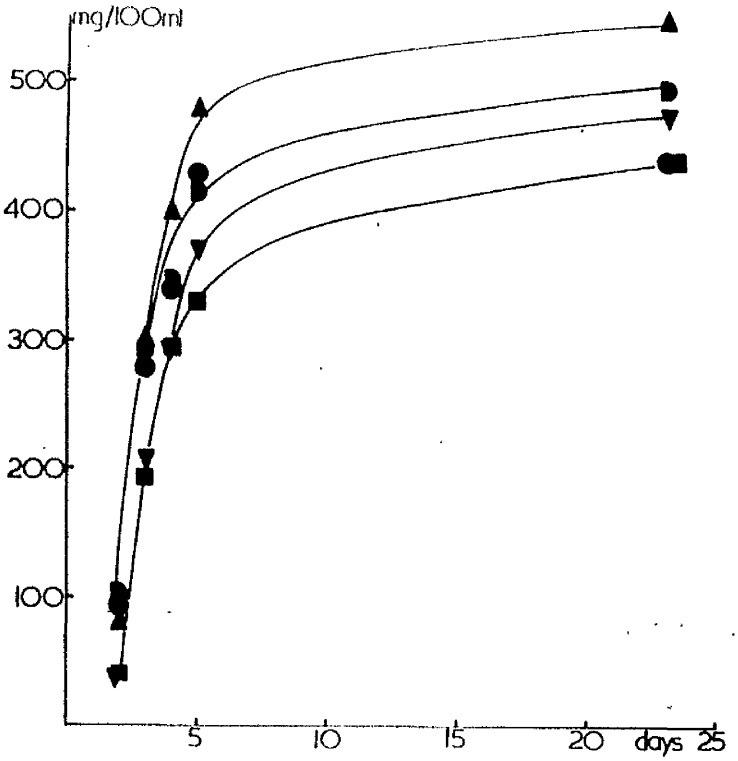


Fig.25 Effect of small amounts of culture filtrate from large-inoculum and small-inoculum stationary cultures of Aspergillus oryzae in advanced stages of growth on the growth pattern of cultures similar, but from large inocula only, when the filtrate was added at inoculation. Filtrates were obtained from the stationary cultures shown in Fig.24.

large-inoculum cultures	stage of growth and type of control culture from which filtrate was taken			
	stage	days	mg/100 ml	type
▣ - ▣	1'	7	76.8	small-inoculum
▽ - ▽	1	7	199	large-inoculum
△ - △	2	14	354	large-inoculum
● - ●	steamed substrate only added (control)			
○ - ○	water only added (control)			

Substrate: as for Fig.24

Table of Fig.25

Statistical analysis

Samples compared		't' actual	't' theoretical	significance
◗ and ▲;	5 day (penultimate samples)	1.72	2.78	not sig.
◗ and ▼;	5 day (penultimate samples)	1.17	2.78	not sig.
◗ and ■;	5 day (penultimate samples)	2.30	2.78	not sig.
◗ and ●;	5 day (penultimate samples)	0.23	2.78	not sig.
▼ and ■;	5 day (penultimate samples)	2.46	2.78	not sig.
◗ and ▲;	23 day (last samples)	1.67	2.78	not sig.
◗ and ▼;	23 day (last samples)	0.45	2.78	not sig.
◗ and ■;	23 day (last samples)	1.69	2.78	not sig.
◗ and ●;	23 day (last samples)	1.67	2.78	not sig.
▼ and ■;	23 day (last samples)	1.11	2.78	not sig.

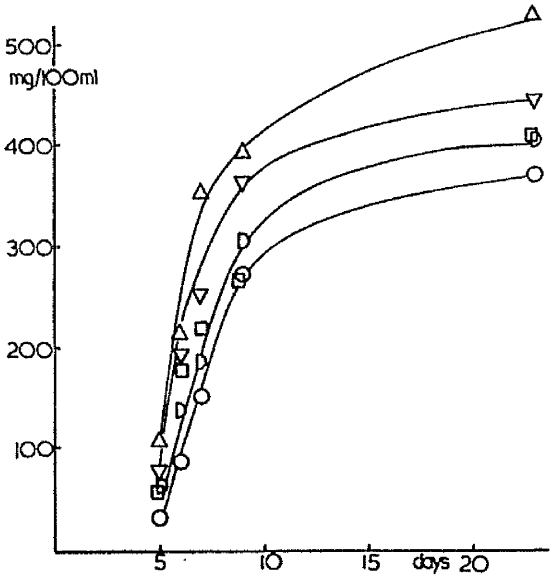


Fig.26 Effect of small amounts of culture filtrate from large-inoculum and small-inoculum stationary cultures of Aspergillus oryzae in advanced stages of growth on the growth pattern of cultures similar, but from small inocula only, when the filtrate was added at inoculation. Filtrates were obtained from the stationary culture controls shown in Fig.24.

small-inoculum cultures	stage of growth and type of control culture from which filtrate was taken			
	stage	days	mg/100 ml	type
□ - □	1'	7	76.8	small-inoculum
▽ - ▽	1	7	199	large-inoculum
△ - △	2	14	354	large-inoculum
D - D	steamed substrate only added (control)			
O - O	water only added (control)			

Substrate: as for Fig.24

Table of Fig.26

Statistical analysis

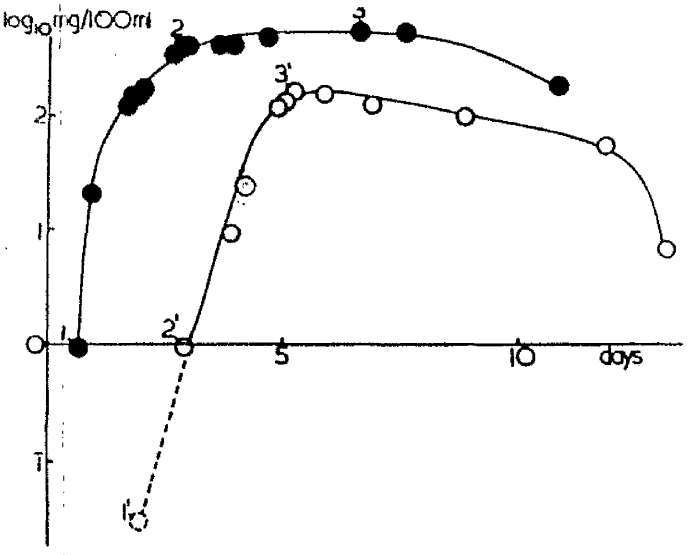
Samples compared		't' actual	't' theoretical	significance
D and △;	9 day (penultimate samples)	2.47	3.18	not sig.
D and ▽;	9 day (penultimate samples)	2.02	2.78	not sig.
D and ○;	9 day (penultimate samples)	0.54	2.78	not sig.
D and □;	9 day (penultimate samples)	1.69	2.78	not sig.
△ and ▽;	9 day (penultimate samples)	0.71	3.18	not sig.
○ and □;	9 day (penultimate samples)	0.11	2.78	not sig.
○ and ▽;	9 day (penultimate samples)	1.55	2.78	not sig.
○ and △;	9 day (penultimate samples)	1.37	3.18	not sig.
△ and D;	23 day (last samples)	6.75	4.60 (1%)	highly sig.
△ and ○;	23 day (last samples)	5.40	4.60 (1%)	highly sig.
△ and ▽;	23 day (last samples)	3.99	2.78	significant
D and ▽;	23 day (last samples)	2.05	2.78	not sig.
D and □;	23 day (last samples)	0.12	2.78	not sig.
D and ○;	23 day (last samples)	1.22	2.78	not sig.
○ and □;	23 day (last samples)	0.92	2.78	not sig.
○ and ▽;	23 day (last samples)	2.49	2.78	not sig.

When the filtrates were added separately to similar cultures grown from large inocula, it was established that addition of filtrates from all stages tested showed no significantly different effect from that of addition of steamed substrate (Fig.25). This conclusion is statistically supported by the Table of Fig.25.

When the effect of such filtrates on small-inoculum cultures was examined, the results shown in Fig.26 were obtained. On making a statistical examination of cultures grown for nine days, no significant difference could be demonstrated between any of 6 pairs tested (see Table of Fig.26). The same cultures at a later stage of growth (23 days) differed significantly from each other in mycelium content in some cases. The mycelium content of the culture which had been supplemented with filtrate from the late-stage large-inoculum culture (stage 2) was greater than that of either of the two control cultures by a highly significant amount (△as compared with ▷ and with ○, Table of Fig.26). The mycelium content of the same culture was significantly greater than that of a culture supplemented with filtrate from an earlier stage of growth (stage 1) of a large-inoculum culture (△as compared with ▽, Table of Fig.26). The indication is that the content of self-stimulatory substances in a large-inoculum culture is considerable and

that it is more pronounced in late stages of culture development than in early stages. No significant difference could be demonstrated between any other of the five pairs chosen for examination at the same stage (Table of Fig.26).

The effect of culture filtrate when added at inoculation to cultures with 100 mg/l EDTA was also examined, and is shown in an earlier figure (Fig.16, p.72). Two culture filtrates were used. One of these was from an identical culture which had already been grown for 13 days (collected at stage 2, Fig.15). It had almost no influence on the growth of the culture (D compared with □, Fig.16). The other filtrate was from a culture which was similar except that it had contained no EDTA, and had already been grown for 13 days (collected at stage 1, Fig.15). This second culture filtrate was stimulatory (▽compared with □, Fig.16). These results show that substances may be produced in the culture which, when introduced at an early stage (with the inoculum in this instance), influence growth at a late stage.



(a)

(b)

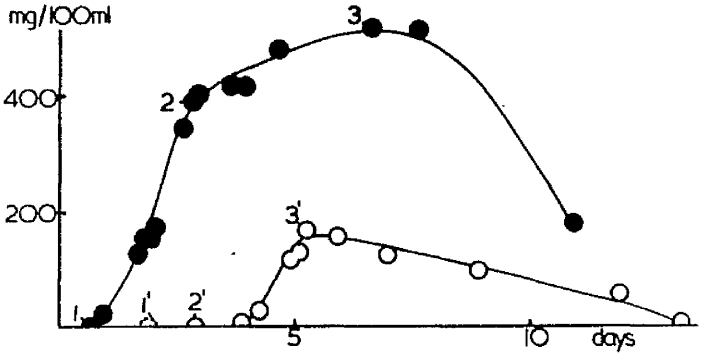


Fig.27 Growth pattern of cultures of Aspergillus oryzae grown in deep culture from large (◉ - ◉) and small (O - O) inocula at high mechanical stress. The results have been plotted semi-logarithmically (Fig.a) and linearly (Fig.b). Filtrates were collected at points 1, 2 and 3 (large-inoculum culture) and at points 1', 2' and 3' (small-inoculum culture)

Substrate: A_3 40 g/l glucose

2332 Self-stimulatory and self-inhibitory substances in deep cultures

Filtrates from deep cultures were also tested. The pattern of growth of the parent culture and the stages from which the filtrates were taken is shown in Fig.27. The striking difference in maximum yield in cultures from large and small inocula should be noted (Fig.27b).

In order to examine the filtrate of a small-inoculum culture at a fairly early stage (46 hour), it was necessary to collect this filtrate from a point where mycelium production was so small that even a large sample from a 5-l culture did not provide enough mycelium for weight determination. An estimated point was therefore designated.

In the linear graph (Fig.27b) the abscissa of the point is governed by the time of sampling, namely 46 h. At this time, production of mycelium was so small that for all practical purposes the point will lie on the line of the horizontal axis. In the semi-logarithmic graph (Fig.27a), the time of sampling (46 h) again determines the abscissa. At this time of sampling, the culture is probably in the phase of exponential growth for reasons adduced by Meyrath (1963) and the point will lie on a line extrapolated backwards from the exponential phase. These two measurements allow the

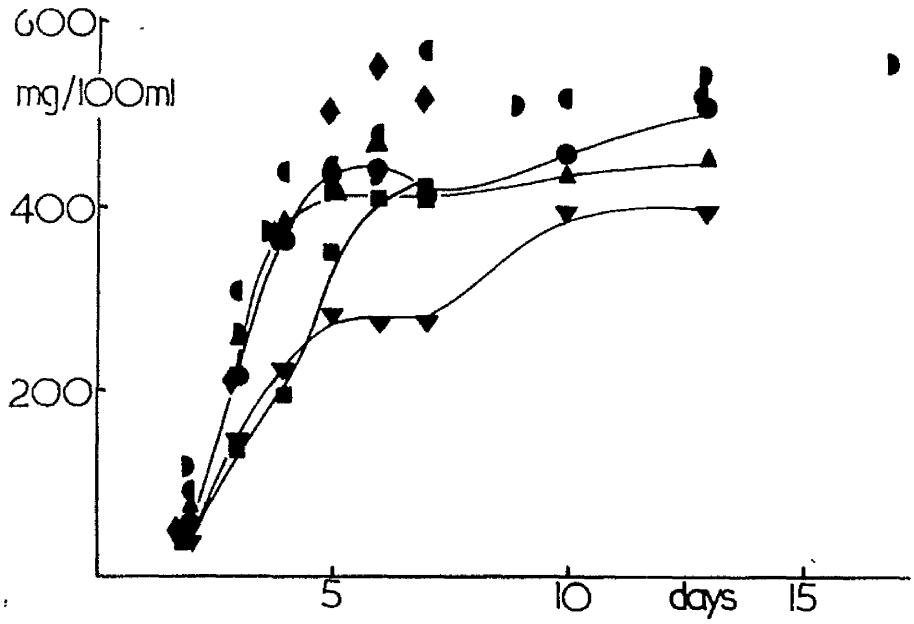


Fig.28 Effect of small amounts of culture filtrate from deep cultures of Aspergillus oryzae on the growth of large-inoculum stationary cultures of the same organism, added at inoculation. The added filtrate had been collected from the large-inoculum culture of Fig.27 at stages 1 (◗ - ◗), 2 (◖ - ◖) and 3 (◈ - ◈) and from the small-inoculum culture of Fig.27 at stages 1' (▽ - ▽), 2' (▣ - ▣) and 3' (△ - △). Control cultures (◎ - ◎) contained no added filtrate.

Substrate: as for Fig.27

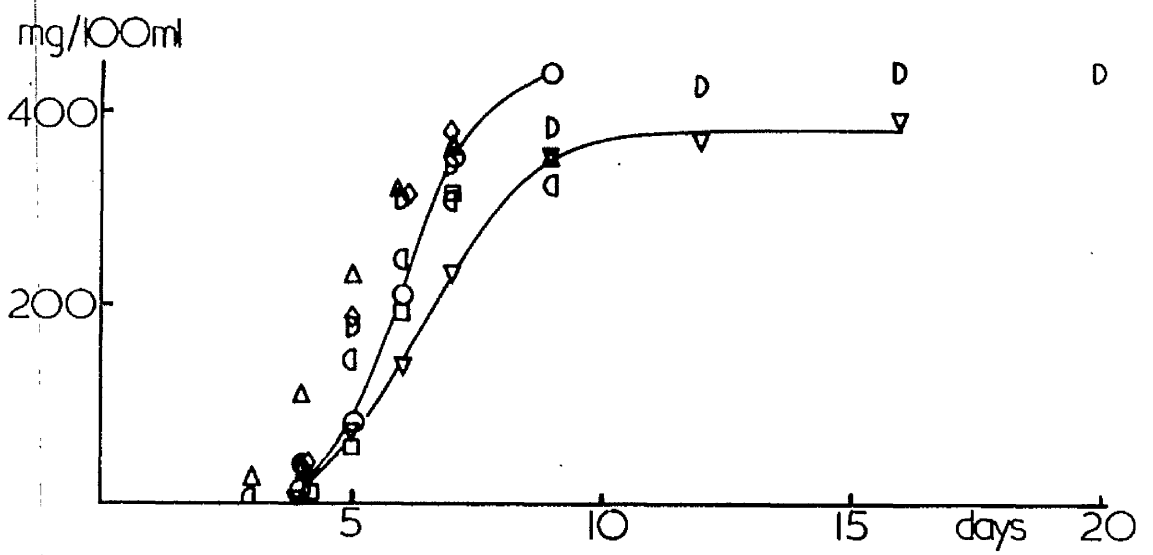


Fig.29 Effect of small amounts of culture filtrate from deep cultures of Aspergillus oryzae on the growth pattern of small-inoculum stationary cultures of the same organism, added at inoculation. The added filtrate had been collected from the large-inoculum culture of Fig.27 at stages 1 (D - D), 2 (◫ - ◫) and 3 (◇-◇) and from the small-inoculum culture at stages 1' (▽-▽), 2' (□-□) and 3' (△-△). Control cultures contained no added filtrate and the results shown (○ - ○) were the average of three sets of results in which water and the original substrates of the deep cultures were tested separately.

Substrate: as for Fig.27

Tables of Figs.28 and 29

Table of Fig.28

Statistical analysis

Samples compared	't' actual	't' theoretical	significance
▼ and ● ; 2 day (1st samples)	6.57	4.60 (1%)	highly sig.
▼ and ● ; 3 day (2nd samples)	2.81	2.78	significant
▼ and ● ; 4 day (3rd samples)	3.18	2.78	significant

Table of Fig.29

Statistical analysis

Samples compared	't' actual	't' theoretical	significance
▽ and D ; 5 day (3rd samples)	3.63	2.78	significant

point to be fixed in the manner shown in Fig.27a. The estimated mycelial content was obtained by reference to the ordinate of the point and was found to be 0.06 mg/100 ml.

Those filtrates were tested against large-inoculum cultures growing in the same substrate but under stationary conditions. It can be seen (Fig.28) that the filtrates from the large-inoculum cultures at stages 1, 2 and 3 all tended to be stimulatory to both growth rate and maximum yield of the culture. In contrast, filtrate from the earliest stage of a small-inoculum culture (1') was inhibitory in action. Filtrate from later stages of small-inoculum cultures differed less from the control cultures (Fig.28). Most weight estimates in this and the following figure had been made singly instead of, as usual, in triplicate. Three pairs for Fig.28 and one pair for Fig.29 could be assessed, however, and comprise the Tables of Figs.28 and 29. Statistical comparison was made of weights after 2, 3 and 4 days' growth of large-inoculum cultures to which had been added filtrate at stages 1 (large-inoculum culture) and 1' (small-inoculum culture) and it can be seen that the inhibitory action of the filtrate from small-inoculum cultures as compared with that from large-inoculum cultures was significant in each case (Table

of Fig.28).

When these filtrates were tested on small-inoculum stationary cultures growing in the same substrate, it can be seen (Fig.29) that addition of filtrates from stages 1, 2 and 3 (large-inoculum culture) gave no clearly-marked effect. Again, however, filtrate from an early stage of a small-inoculum culture tended to be inhibitory to both growth rate and maximum yield. A 5-day culture containing filtrate from the earliest stage of a small-inoculum culture had a significantly lower mycelium content than did a similar culture containing filtrate from a large-inoculum culture (∇ and D , Table of Fig.29).

24 DISCUSSION

Preliminary experiments had shown that size-of-inoculum effects might be expected under a range of conditions. An effect might show as a difference in rate of growth at early or late stages, or as a difference in maximum yield, between cultures from large and small inocula. Effects in preliminary experiments were less marked than had been anticipated. As was stated "The pronounced effect of size of inoculum shown in the substrate without trace elements had been obtained in experiments in Zürich. A repetition of these experiments in Glasgow gave less marked results." (McIntosh & Meyrath, 1964b). An example of the Zürich results is shown in Fig.1 of the same publication and, particularly when trace elements are absent, is illustrative of what has been called the 'normal' effect of inoculum size, namely, higher rate of growth in the linear phase and higher maximum yield in a large-inoculum culture. The conclusion was drawn (McIntosh & Meyrath, 1964b) that since "trace elements were known to reduce the effects it was probable that trace elements present in the constituents of the substrate were responsible for the diminished response." Such a conclusion was a logical basis for extended work on

trace elements and led to attempts to block them, to remove them from glucose by a purification procedure or to examine their effect when added singly or in particular combinations. Increased growth rate and/or maximum yield in trace-element-poor substrates when a large inoculum is used is not due simply to the transfer of large amounts of trace elements. Meyrath (1963) has shown that although the ash of the conidia from a large inoculum, or a large inoculum of dead conidia was added to a small-inoculum culture, the growth of the culture was not thereby made similar to that of a large-inoculum culture. Moreover, a culture from an 'extremely small' inoculum could give a higher yield than a culture from a large inoculum (Meyrath, 1963).

Of the individual trace elements added, Ca and Cu had the most pronounced effect, with a tendency for Ca to increase the effect of inoculum size and Cu to abolish or even reverse it. Ca has already been shown to emphasize the effect of size of inoculum (Meyrath & Ng, unpublished).

When EDTA was used, it was not found possible to produce a sufficient difference in growth between cultures from large and small inocula without abolishing the growth of the small-inoculum culture. The results did, however, point to a possible mechanism for the effects of inoculum

size. The fact that small-inoculum cultures were not able to overcome the action of EDTA as were large-inoculum cultures indicates that the latter were excreting substances which counteracted the influence of EDTA. This counteraction can probably best be explained by assuming that these substances are also chelating agents which compete with the EDTA for the trace elements available but which form complexes capable of being assimilated by the cell; thus trace elements are transported into the cell. Obviously a few conidia would be less able to excrete these substances in a concentration sufficient to compete with the EDTA than would a large number of conidia.

The $Al(OH)_3$ treatment of glucose under certain conditions produced a 'reversal' phenomenon. A reversal phenomenon has already been reported when the carbohydrate used was maltose, at a concentration of 80 g/l (Meyrath, 1963). These findings are a further indication of the inadequacy of a 'transfer' theory to explain the inoculum-size effect in absence of trace elements.

The action of Zn, though not studied in detail, is of interest. It was stated (McIntosh & Meyrath, 1964b) "Instances where a low concentration of a trace element stimulates and a high concentration inhibits are relatively

commonplace but in this case low concentrations are inhibitory and very low as well as higher concentrations are, in comparison, stimulatory." Striking confirmation of the occurrence of unusual concentration effects such as that described for Zn has come in a recent publication by Schatz, Schalscha & Schatz (1964). These workers have collated such reports scattered through many fields and termed them "paradoxical effects" They write "These effects have ... been looked upon as isolated cases. Actually, however, they comprise a distinct group of dose-response reactions, each including a concentration range characterized by more inhibition at the lower level. The unique aspect of the paradoxical effect is that inhibition or toxicity is overcome by simply adding more of the original inhibitor or toxic agent and nothing else. In other words, more of the same thing produces a diametrically opposite effect; that is, a purely quantitative change in one constituent transforms the system qualitatively." The same workers suggest several mechanisms for this type of effect. For example, it is possible that certain enzymes in an integrated biochemical pathway vary in susceptibility to an inhibitor or to intermediate products accumulating as a result of inhibition. Again, the effects may be due to the formation of a series

of metal complexes with members of the series differing in biological properties.

Purification of the glucose with subsequent addition of trace elements singly or in groups gave further indication of the role of trace elements. Fe gave a reversal effect when it was the only trace element present. This means that the fact that Fe was included in the basal medium for so many of the experiments had probably contributed to a diminishing of the effect of inoculum size.

It was further shown that caramelization gave rise to substances with an effect on culture growth. Sergeant, Lankford & Traxler (1957) pointed out the stimulatory effect obtainable for certain Bacillus species by adding glucose sterilized together with phosphate. Lankford, Kustoff & Sergeant (1957) tested a variety of chemical compounds for their capacity to substitute for autoclaved glucose-phosphate solution in stimulation of growth initiation of E. globigii. Almost all of the active compounds possessed structural characteristics known to confer metal chelating potential. White (1964) in spectrophotometric studies in the U.V. range showed that whereas solutions of uncaramelized sugar (maltose and glucose) did not have any absorption spectrum at the dilutions used, the caramelized sugar solutions had characteristic absorption maximum at

285 mμ. This is in the range over which carbonyl groups absorb and these are known to exert a complexing action. In fact this absorption peak could be shown to disappear after addition of salts of heavy metals. White suggested therefore that these caramelized products were of a chelating nature.

In this work, self-inhibitory substances have been demonstrated in cultures of Aspergillus oryzae, particularly in the very early stages of small-inoculum cultures. Self-stimulatory substances have also been shown to be produced and these are found particularly in later stages of large-inoculum cultures. It has been shown in various ways that these substances are not simply transferred with an inoculum, but are produced in the growing culture. It has been shown moreover that small-inoculum cultures advanced in age possess stimulatory substances, but these do not act although sufficient nutrients remain in the substrate to permit enhancement of growth to occur. The conclusion to be drawn seems to be that substances in small-inoculum cultures which have been formed at very early stages of growth have so adversely affected the mycelium that the later production of stimulatory substances cannot reverse the effect. It may well be the efficiency of assimilation

which is irreversibly affected, because small-inoculum cultures have been shown to assimilate carbon and nitrogen from given sources less efficiently than large-inoculum cultures (section 3). It can be inferred that these stimulatory substances are non-volatile because they were retained in the substrate after it had been steamed. They therefore differ from some of those described in other fungi and mentioned above.

Kojic acid has a well-known effect as a chelating agent and is often produced by strains of Aspergillus flavus-oryzae (see Foster, 1949). Under the conditions of these experiments, however, the strain used in this work lacked the ability to produce Kojic acid as indicated by the ferric chloride test.

Vikén and Agthe (1958), in studies on Saccharomyces cerevisiae, showed that β-alanine was less able to counteract the inhibitory action of biotin when the β-alanine was added after 78 h of incubation than if added at the stage of inoculation. Biotin added at an early stage thus had an adverse effect on late stages of growth. β-alanine added at an early stage had a greater influence than when added at a late stage. The importance of age of culture in producing cells showing a negative Pasteur effect has been

shown by Wikén and co-workers; no negative Pasteur effect was obtained with cells from old cultures of either Saccharomyces carlsbergensis (Pfennig & Wikén, 1960a) or certain strains of Brettanomyces (Wikén, Scheffers & Verhaar, 1961).

Cumulative evidence therefore shows that inoculum size phenomena depend in no small measure on self-stimulatory and self-inhibitory substances produced, and that the stage at which these are produced is of critical importance (see also Meyrath & McIntosh, 1965).

SECTION 3

METABOLISM OF CARBON AND NITROGEN

3 METABOLISM OF CARBON AND NITROGEN

31 INTRODUCTORY

In this work, which was concerned with Aspergillus oryzae and Aspergillus niger, the fungi were supplied with starch or glucose as source of carbon and ammonium sulphate as source of nitrogen. Below, methods are described for assessing the extent of their utilization so that the metabolic efficiencies of various cultures may be determined. Other aspects of carbon metabolism such as respiratory quotient are also considered. Porter (1946) listed the nitrogen contents of a large number of micro-organisms and showed the range of nitrogen content in bacteria to be from 8 - 15%. Foster (1949) considered that filamentous fungi did not have complex nitrogenous requirements such as were necessary for the reproduction of many bacteria. He considered that ability to utilise nitrogen in the form of nitrate represented greater versatility on the part of a fungus than did ability to utilise this element in the form of ammonia, because all nitrate users could utilise ammonia but not vice versa. This is in contrast to the higher plants for which nitrate is the preferred nitrogen source.

In this study on the utilization of nitrogen, its incorporation into the mycelium of the mould as organic-N, and its excretion in an organic form are considered. Formation of extra-cellular nitrogenous material is not simply the result of autolysis or senescence. It can take place in a developing culture to a considerable extent, as was shown in Scopulariopsis brevicaulis by Morton and Broadbent (1955) and in Aspergillus oryzae by Crowther and Lennox (1953).

32 MATERIALS AND METHODS

321. Growth Procedures

All Figures in this section, with the exception of Figs. 30 and 32, show the results of a study of the metabolic properties of cultures for which growth patterns have already been described in Section 2. In the legend for each of these figures, a cross-reference has been made to the relevant Figure in Section 2 which gives this growth pattern; reference should also be made to that Section for information concerning the materials and methods employed.

Fig.32 (p.124) shows the results of the only respiration experiment performed on stationary cultures. Since it was desired to examine an early stage of growth when mycelial content was low, fairly large volumes of substrate were required. This substrate was A_4 (Table A, facing p.34), as used for respiration studies of deep cultures. The substrate was distributed in 100-ml flasks in 25-ml amounts to which 1 ml of a suspension containing 0.5×10^7 (large inoculum) or 10^3 (small inoculum) conidia was added so that the final concentration of conidia was 2×10^7 per 100 ml (large inoculum) or 4×10^3 per 100 ml (small inoculum), i.e. the same concentrations as were used for deep cultures. The flasks were incubated at 25^0. The contents of 10 flasks of each kind of culture were bulked to give 2 flasks of 250 ml each, one for each size of inoculum. A few ml of these bulk samples were used in respiration experiments and the remainder was used for estimation of mycelium dry weight. The methods of estimation of oxygen uptake and carbon dioxide output are as given in Section 4 (p.138).

322. Estimation of utilization of starch

The method used to estimate starch was basically a method for the estimation of reducing sugars (see Stiles, Peterson and Fred, 1926). It was necessary to construct a reference curve for the estimation of sugar and also to hydrolyse the starch to produce reducing sugars.

Reference curve : For the preparation of the reference curve exactly 1 g of glucose of analytical reagent quality was dissolved and made up to 100 ml in water. From this solution, dilutions were made containing, in turn, 40, 20, 10 and 4 mg/100 ml. Portions of these sugar solutions (5 ml), together with 5 ml copper reagent (Stiles, Peterson & Fred, 1926) were mixed in boiling tubes covered with aluminium foil. The tubes were placed in a boiling water-bath for 15 min then cooled in tap water to approximately 20^{0}. Five ml of 1 N sulphuric acid were added to each tube and the mixture shaken or stirred by a spiral-ended stirring rod for exactly one minute. The vigorous evolution of CO_2 removed traces of oxygen which would interfere with the iodometric estimation. The mixture was then titrated immediately with 0.005 N thiosulphate solution. When the brown colour of the iodine nearly

vanished, 0.5 ml of 1% starch solution (preserved by adding 0.5% phenol) was added and the titration completed. The end point was shown when no clouds arose on stirring after addition of thiosulphate. The final colour of the solution was greyish-blue. The 0.005 N thiosulphate was made afresh on every day of using by diluting stock 0.1 N sodium thiosulphate solution 1 in 20. A blank of copper reagent with no sugar added was performed in each case and the difference between the blank and the test was taken as the final reading.

Hydrolysis: It was considered advisable to ascertain the optimal conditions for the hydrolysis of starch since the substrate contained considerable amounts of phosphates as buffering agents, a factor which does not normally have to be taken account of in such estimations. Further, it is not always clear whether the conditions of hydrolysis lead to exact quantitative recovery of the glucose produced, as there may be partial decomposition of the sugars as well as formation of reversion products, i.e. oligosaccharides of a kind other than starch, such as isomaltose.

The method finally adopted for examination was as follows: 2 ml conc HCl (≏10N) were added to 5 ml culture filtrate and diluted to 20 ml with distilled water to give

a final HCl concentration of 1 N. The solution was steamed in 50-ml conical flasks for 1 h and then cooled quickly to avoid evaporation. The solution was made slightly alkaline to litmus paper with 10 N NaOH then made up to a known volume depending on the concentration of the sugar. Duplicate analyses were performed on a blank using water instead of sugar solution, on a sample of known concentration of glucose as a control and on each sample of filtrate, using the procedure described for determination of the reference curve.

323. Determination of pH and titratable acidity

The pH value of filtrates was determined by use of a glass-calomel electrode system. Titratable acidity was determined by titrating aliquot portions of the filtrate against NaOH solution (phenolphthalein indicator).

324. Estimation of Nitrogen

Digestion: When filtrates or pellets of dried mycelium required to be digested, the material was introduced into a

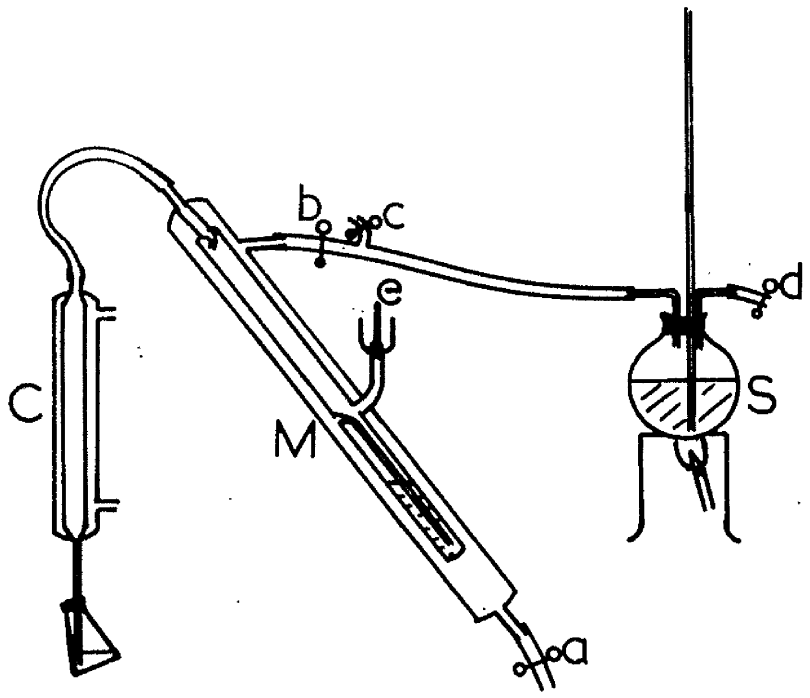


Fig.30 Distillation apparatus for estimation of Nitrogen

C - Condenser

M - Markham still

S - Steam generator (proportionately reduced in size)

small Kjeldahl flask together with a few crystals of cupric sulphate and a knife point of selenium, and 2 ml conc. H_2SO_4 added. The mixture was boiled gently until the solution was clear, indicating that all the nitrogen present was in the form of ammonium sulphate. This process took up to 20 h.

Steam distillation: The apparatus was as shown in Fig.30. In the receiving flask was placed 20 ml boric acid solution (40 g/l) containing 4 drops methyl red indicator. The sample was steam-distilled in presence of excess NaOH until a volume of about 50 ml had distilled over into the recovery flask, at which point the contents of the flask were removed for estimation.

The boric acid solution containing the trapped distillate was titrated with sulphuric acid. A blank was always performed in which the procedure was identical, but only steam was distilled over. The value for this blank was always allowed for in the final calculation.

Three aspects of nitrogen metabolism were examined, namely, total nitrogen content of the mycelium, organic nitrogen in the filtrate and ammonia nitrogen utilised. The value for total nitrogen content of the mycelium was

obtained by subjecting the pellets of mycelium, of known dry weight, to digestion followed by nitrogen estimation as above. The nitrogen content in relation to mycelium dry weight could therefore easily be obtained. The value for organic nitrogen present in the filtrate was calculated from the difference between the estimate for total nitrogen (using digested filtrate) and the estimate for inorganic (residual ammonia-) nitrogen (using undigested filtrate). The value for ammonia nitrogen utilized was obtained by a difference method. A nitrogen estimation was performed on the original substrate, to obtain a value for ammonia nitrogen available. Similarly, a nitrogen estimation was performed on the undigested filtrate, to obtain a value for ammonia nitrogen remaining. The ammonia nitrogen utilized was given by the difference between these two values.

Table B

Relationship of rate of oxygen uptake and respiratory quotient (RQ) of mycelium to stage of growth and age of large-inoculum cultures of Aspergillus oryzae.

large-inoculum cultures of Aspergillus oryzae.

	Stage of growth (mg dwm * /100 ml)	Age of culture (h)	Rate of uptake of O_2 (µl/ mg dwm*.h)	RQ
a)	8.7	22	153	1.11
b)	16.6	25	190	1.03
c)	229.2	48	28	1.39
d)	310.8	96	30	1.03
e)	52.0	191	35	1.03

* dwm dry weight mycelium

Substrate A_4. Mycelium suspended in culture filtrate and glucose. The growth curve of this experiment is given in Fig.7 (closed circles).

33 RESULTS

331. Q - metabolism

Several reports in the literature have shown that specific rate of oxygen uptake (oxygen uptake/unit weight cell material and unit of time) by micro-organisms decreased as the age of the culture increased (Tamiya, 1942; Hockenhull, Fantes, Norbert & Whitehead, 1954). Of the first three stages of growth shown in Table B (a, b and c), it is probable that a and b were within the phase of exponential growth and c just beyond it. During this time it might have been expected that the rate of oxygen uptake would have remained fairly constant, yet it decreased from 190 to 28 µl/mg h. This, it should be noted, was in a large-inoculum culture. It was shown (Meyrath & McIntosh, 1963b) that small-inoculum cultures gave mycelium showing low rates of oxygen uptake (55 or 57 µl/mg h) at all stages of measurable growth; large-inoculum cultures under the same conditions gave a decreasing rate of oxygen uptake (150 to 100 to 56 µl/mg h) even within the phase of exponential growth.

It appears that mycelium from small-inoculum cultures

at all stages of measurable growth has the properties of mycelium of large-inoculum cultures at late stages of growth. The most likely explanation is that small-inoculum cultures undergo an aging process (leading to a decrease in uptake of oxygen) during the prolonged time they take to reach the same content of mycelium as is found in large-inoculum cultures at early stages of growth. The decrease in oxygen uptake during exponential growth in a large-inoculum culture means that less oxygen was required to form a given weight of mycelium at later stages of growth. If the amount of energy required to synthesise unit weight of mycelium is constant during exponential growth, and if a given amount of oxygen taken up releases a proportional amount of energy for cell synthesis, part of this energy must be furnished by a procedure which is unaccompanied by oxygen uptake. The suggestion that energy in such circumstances might be forthcoming from anaerobic processes has already been made in connection with anaerobic spoilage of fruit juices by moulds (Meyrath, 1962b). Consequent upon this anaerobic metabolism is an inefficient utilization of the carbon source. Since small-inoculum cultures have a lower specific rate of oxygen uptake during measurable growth, they may be expected to utilize the carbon source less

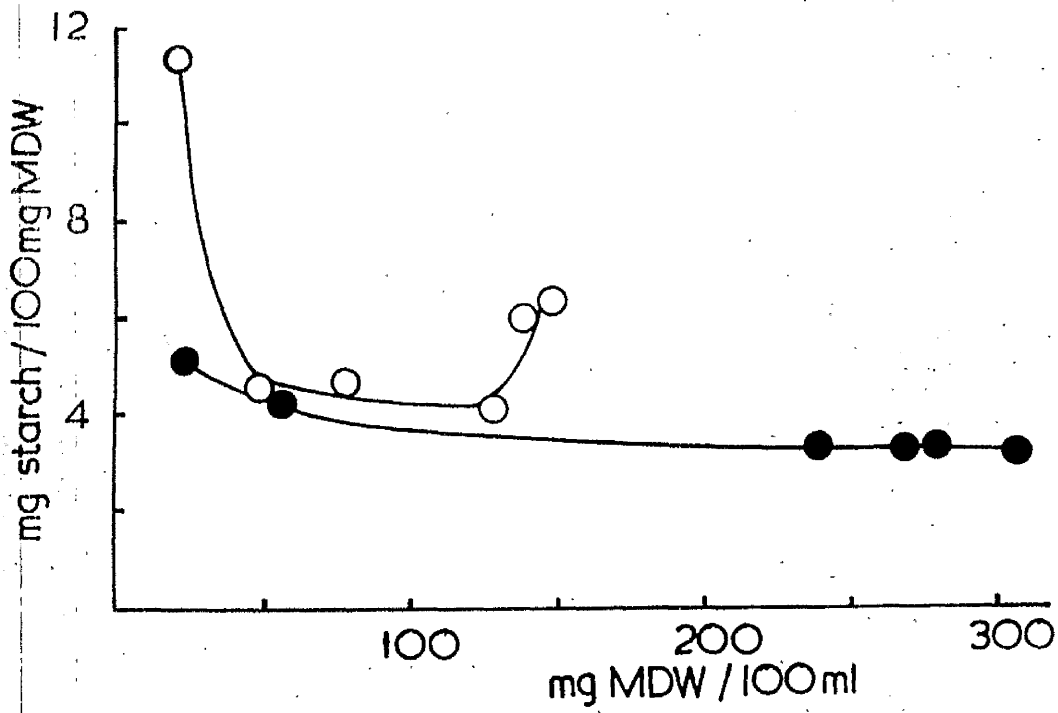


Fig.31 Specific utilization of starch by Aspergillus oryzae in deep cultures at various stages of growth from large (● - ●) and small (O - O) inocula. The growth curves of the cultures from which these samples were taken appear in Fig.7 (triangles).

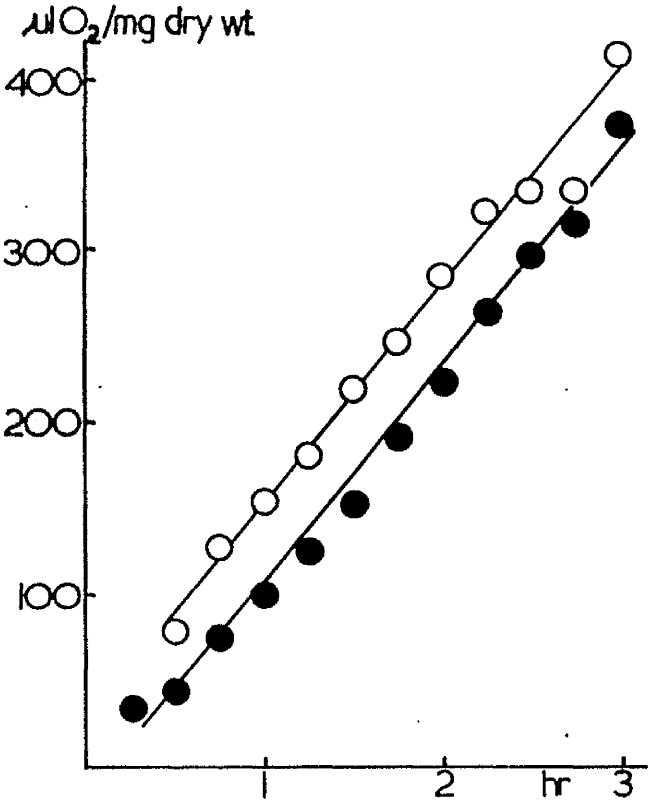


Fig. 32 Uptake of oxygen in large-inoculum and small-inoculum cultures of Aspergillus oryzae grown under stationary conditions. The large-inoculum (● - ●) culture samples were taken after 44 h and had a mycelium content of 4.6 mg dry wt/100 ml. The small-inoculum (O - O) culture samples were taken after 88 h and had a mycelium content of 2.4 mg dry wt/100 ml.

efficiently than do large-inoculum cultures. That they in fact do so is shown in Fig.31 which shows that there is a less efficient utilisation of the carbon source by a small-inoculum culture at all stages of growth. In both cultures all the starch was metabolized at the point where maximum yield was reached. The outcome of these inter-related factors is that a large-inoculum culture gives a higher maximum yield of mycelium than does a small-inoculum culture. Anaerobic dissimilation in older cultures is indicated by an increase in respiratory quotient at some stages of growth (Table B).

An interesting effect is shown in Fig.32 which shows the results of respiration experiments on stationary cultures. The use of stationary cultures was exceptional; other respiration experiments in this work were performed on deep cultures. Fig.32 shows that the rate of uptake of oxygen was almost the same for large-inoculum and small-inoculum cultures. Reasoning from the above, it might have been expected that the small-inoculum culture would give a smaller specific rate of oxygen uptake. In the case of stationary cultures with the trace element combination used there tends to be, however, little effect of inoculum size; that is, large-inoculum and small-inoculum cultures show a fairly

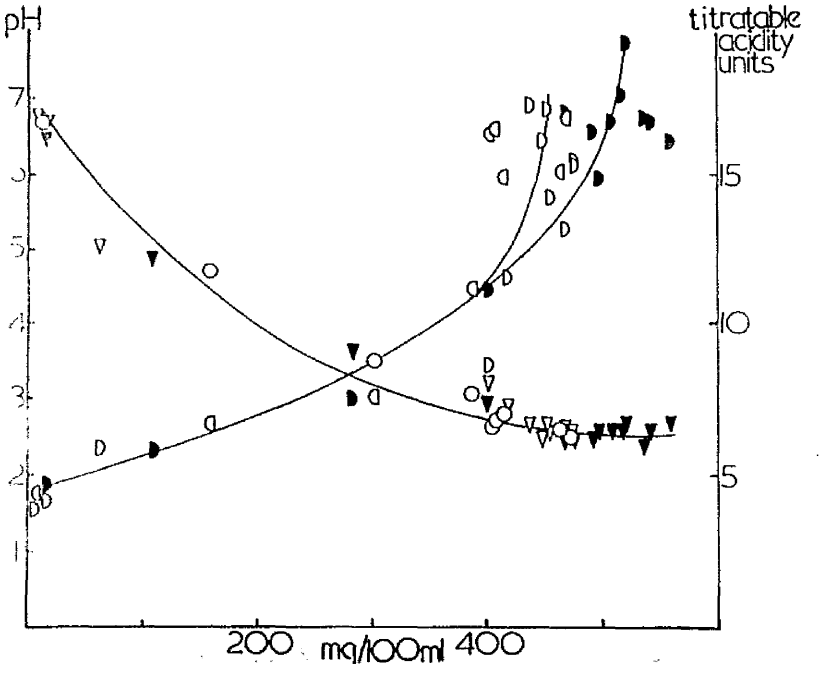


Fig.33 pH value and titratable acidity of cultures of Aspergillus niger from various inocula grown under stationary conditions. The growth curves of these cultures appear in Fig.6.

pH value	size of inoculum from which culture was grown	titratable acidity
▽ - ▽	large	D - D
▽ - ▽	small	D - D
○ - ○	extremely small	D - D

similar growth pattern (e.g. Fig.5, p.45). Bearing this in mind, no difference between the cultures in rate of oxygen uptake should be expected as in fact is the case although oxygenation of the culture is presumably adequate because rate of multiplication at this stage is as high as with deep (aerated) cultures.

This phenomenon was also seen in work with Aspergillus niger, the results of which are given in Fig.33. During growth of A.niger there was a steady increase in titratable acidity and a decrease in pH value. It can be seen however that these values are very similar, except perhaps at late stages of growth, regardless of whether a large inoculum, a small inoculum or an extremely small inoculum (all of conidia) was used (Fig.33). The dissimilatory metabolism as reflected in the production of acids, both inorganic and organic, is thus little different. The cultures from which these filtrates were obtained have already been shown, however, to have little effect of size of inoculum when large, small and extremely small inocula were used, except at late stages of growth (Fig.6, p.51). Elsewhere (Moyrath & McIntosh, 1963b) it has been shown that in cultures with typical effects of inoculum size such as decreased growth rate and decreased maximum yield with small inocula, the

production of organic acids at the various stages of growth was higher in small-inoculum cultures. Since the production of organic acids is due to a low-energy-yielding process as compared with complete oxidation to carbon dioxide, the same conclusion was reached as with those studies related to oxygen uptake and carbon dioxide output, namely, that a small-inoculum culture is less efficient than a large-inoculum culture in its use of available carbohydrate. Studies on the production of alcohol (Meyrath & McIntosh, 1963b) also confirmed this conclusion. The present work using Aspergillus niger (Fig.33) shows the corollary to be true; that is, in the absence of an effect of size of inoculum in respect of growth rate and maximum yield, there was an absence of metabolic differences between cultures from different sizes of inoculum in at least two respects, as shown by considerations of pH value and titratable acidity.

332. N - metabolism

The results of experiments performed on metabolism of N are shown in Figs.34, 35, 36 and 37. It should be remembered that the growth curves of these experiments had shown that the

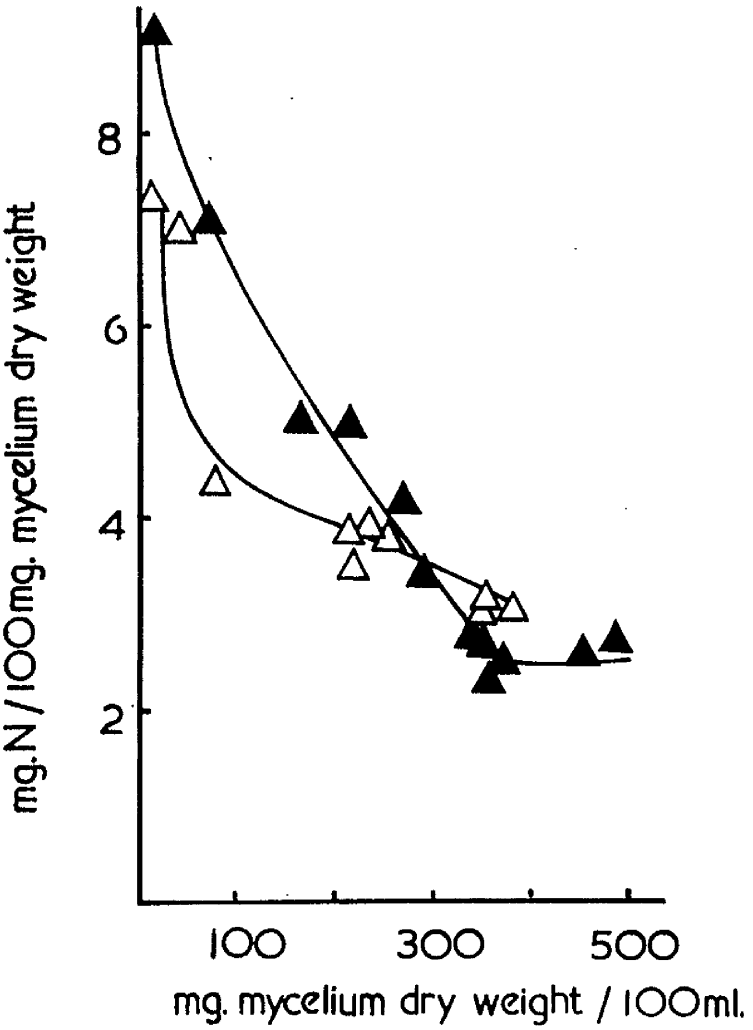


Fig.34 Nitrogen incorporated by Aspergillus oryzae in stationary large-inoculum (▲-▲) and small-inoculum (△-△) cultures in a substrate with added trace elements. The growth curves of the cultures from which the mycelium samples were taken appear in Fig.5 (circles).

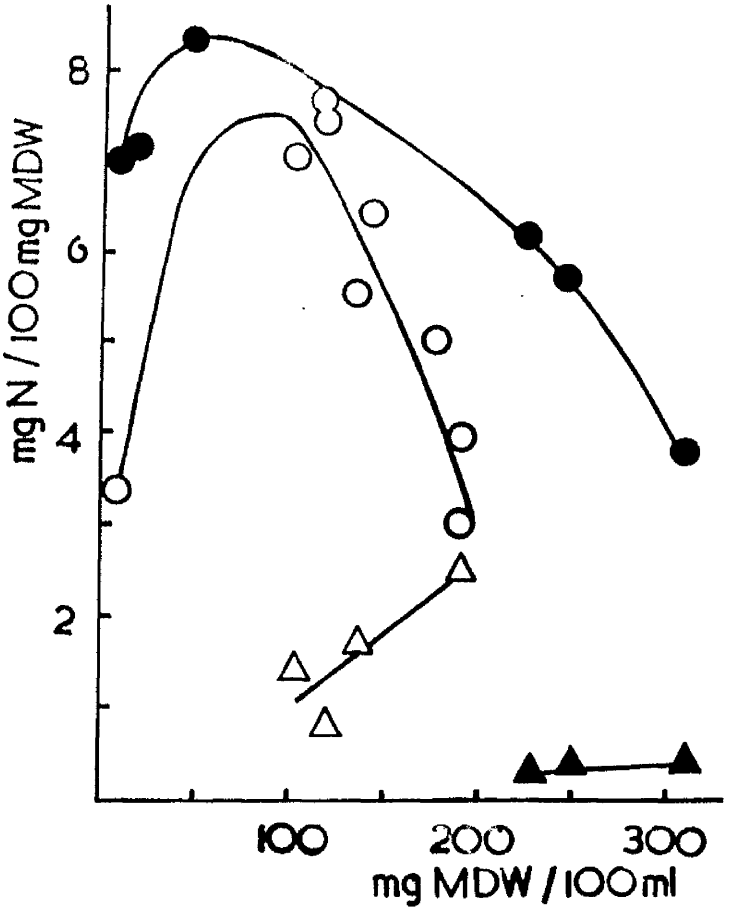


Fig.35 Nitrogen incorporated and excreted by Aspergillus oryzae in deep culture at low mechanical stress. Organic-N in mycelium (N incorporated) was estimated in both large-inoculum (●-●) and small-inoculum (○-○) cultures. Organic-N in filtrate (N excreted) was also estimated in both large-inoculum (▲-▲) and small-inoculum (△-△) cultures. The growth curves of the cultures from which these estimations were made appear in Fig.7 (circles).

maximum mycelial yield was greater when a large inoculum was used and that the difference in maximum mycelial yield as between large-inoculum and small-inoculum cultures was more pronounced when the mechanical stress was increased.

3321 Organic nitrogen in mycelium and in filtrate.

The nitrogen content of the mycelium from stationary cultures was examined first. Under these conditions of no mechanical stress, the nitrogen content of the mycelium in a large-inoculum culture was shown to be higher than that in a small-inoculum culture (Fig.34). The effect is not pronounced; indeed, the curves can be seen to merge and overlap at stages of growth with a high mycelium content.

The results of experiments performed in deep culture at low mechanical stress are shown in Fig.35. In the early stages of growth, a large-inoculum culture (closed circles) gave mycelium with a high nitrogen content which quickly increased to a maximum then slowly decreased. In a small-inoculum culture, the nitrogen content of the mycelium was considerably lower in early and late stages

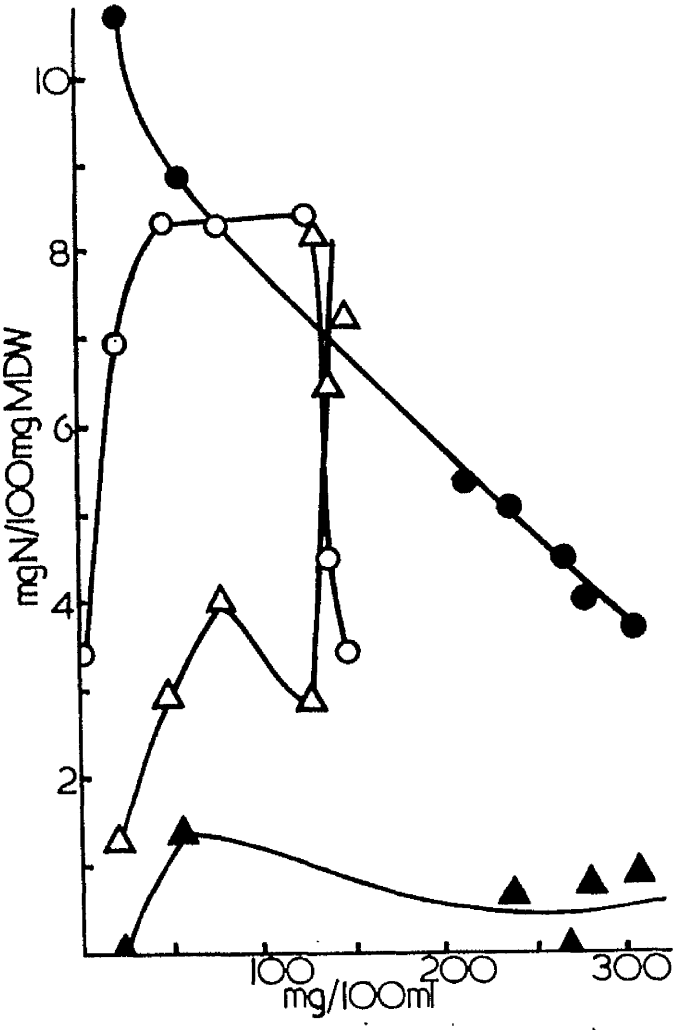


Fig.36 Nitrogen incorporated and excreted by Aspergillus oryzae in deep culture at high mechanical stress. Organic-N in mycelium (N incorporated) was estimated in both large-inoculum (◎ - ◎) and small-inoculum (○ - ○) cultures. Organic-N in filtrate (N excreted) was also estimated in both large-inoculum (▲- ▲) and small-inoculum (△- △) cultures. The growth curves of the cultures from which these estimations were made appear in Fig.7 (triangles).

and probably showed a lower maximum. The amount of organic nitrogen in the filtrate was lower for a large-inoculum culture than for a small-inoculum culture.

These results at low mechanical stress indicate the following pattern of N metabolism. The mycelium from a large-inoculum culture is the more efficient, since it assimilates more N. Moreover, since organic-N in the filtrate represents N excreted by the mould (for inorganic-N only was present in the initial substrate), the results show that a large-inoculum culture excretes very little N. Conversely, in a small-inoculum culture, the lower N content of the mycelium and the higher excretion of organic-N indicate inefficiency in utilization and assimilation of N.

The results for corresponding experiments at high mechanical stress are given in Fig.36. The mycelium at various stages of growth was examined for content of organic-N. The results for the large-inoculum culture corresponded very closely to those shown in Fig.35. The mycelium of the small-inoculum culture showed a decrease in N content, beginning at about the same point (130 mg. dry weight mycelium /100 ml) as when low mechanical stress was used, but the decrease was much more pronounced at high stress.

The filtrate was examined for content of organic-N and the amount was shown to be smaller in a large-inoculum culture as had been found at low mechanical stress. In a small-inoculum culture there was an increase in organic-N in the filtrate as at low mechanical stress, but the increase was more pronounced (Fig.36). Thus under high mechanical stress, as under low, the pattern of assimilation and excretion of N indicates that the large-inoculum culture was the more efficient. Moreover, at a given point, the mycelium of a small-inoculum culture underwent a marked change in metabolic behaviour, evidenced by a rapid and voluminous excretion of N. This occurred while the mycelium content of the culture was still increasing, at a point just prior to maximal growth. It did not take place during what is usually called the phase of decline or the autolytic phase. A comparison of Figs.35 & 36 shows that increased mechanical stress accentuated these differences in the behaviour of large-inoculum and small-inoculum cultures. The overall pattern seems to be that as mechanical stress is increased, so the inability of the mycelium of a small-inoculum culture to incorporate nitrogen becomes more pronounced.

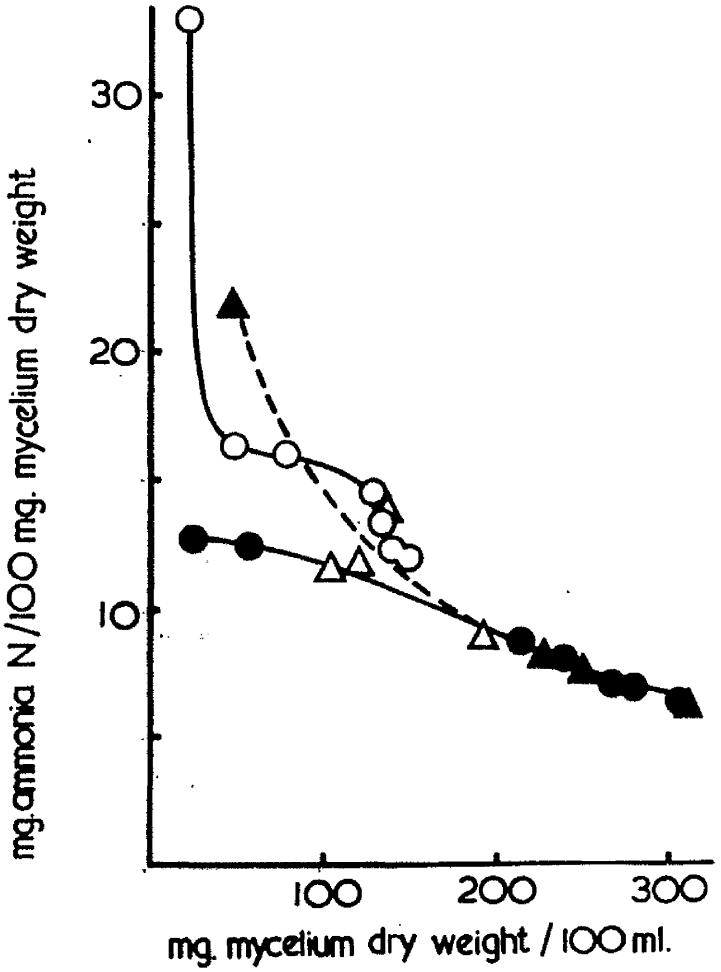


Fig.37 Ammonia nitrogen metabolized by Aspergillus oryzae in deep culture at low and high mechanical stress. The values for large-inoculum (▲-▲) and small-inoculum (△-△) cultures at low mechanical stress are shown by the broken line. The values for large-inoculum (● - ●) and small-inoculum (O - O) cultures at high mechanical stress are shown by the continuous lines. The growth curves of the cultures on which these estimations were made appear in Fig.7.

3322 Metabolism of ammonia-N

At low mechanical stress, there was little distinction between a large- and a small-inoculum culture in the specific amount of ammonia-N metabolized, as is shown in Fig.37. When mechanical stress was increased, however, the small-inoculum culture used specifically more ammonia-N than did a large-inoculum culture. The efficiency of a culture cannot safely be gauged from this aspect of N metabolism; efficiency in metabolism of nitrogen should perhaps be measured not so much by the amount of nitrogen utilized to produce a given weight of mycelium as by what is done with the nitrogen so utilized. Fig.37 does however show that by altering the mechanical stress, the effect of inoculum size on the amount of ammonia-N utilized was also altered. At low mechanical stress there was no evident influence of inoculum size; at high mechanical stress, the small-inoculum culture metabolized more ammonia-N than did the large-inoculum culture.

34 DISCUSSION

It has been shown earlier in this work and elsewhere (Meyrath, 1963; Meyrath & McIntosh, 1963b; McIntosh & Meyrath, 1963a, 1963c, 1964a) that by varying size of inoculum, cultural characteristics such as rate of growth and maximum yield are altered. In conditions where such effects have been found, studies of O and N metabolism have shown that growth effects are accompanied by demonstrable changes in metabolism. Conversely, it has also been shown that where variation in inoculum size was not reflected in growth rate and maximum yield, neither was it reflected in metabolic characteristics such as oxygen uptake, acid production and N content.

It is convenient to discuss here the question of the use of total cellular nitrogen as a determinant for growth. Cochrane (1958) thought dry weight might be deceptive because it represented accumulated polysaccharides, lipids or wall materials without increase in "living protoplasm". But if dry weight is invalidated as a measure of growth by the possible presence of storage carbohydrate or cell wall material, the use of total nitrogen as a measure of growth is likewise invalidated by the possible presence of

nitrogenous storage material. In the present state of knowledge it is difficult to make quantitative distinction between "living protoplasm" nitrogen or carbon and "storage" nitrogen or carbon. In any case it is doubtful whether the distinction should be made for the purpose of assessing growth and the proportion of assimilation as a whole. Material which has been processed and stored by the cell can reasonably be viewed as part of the cell and reckoned as 'growth', since an energy-consuming assimilation process is involved. Production of extra-cellular polysaccharide may be in a different category, but there has been no obvious occurrence of this in these experiments. In support of the value of nitrogen content as an index of growth, both Gunnalus (1951) and Stephenson (1949) reproduce a graph by Hershey & Pronfenbrenner (1938) which shows that metabolic activity (as represented by oxygen uptake) per g nitrogen is fairly uniform throughout a period of 24 hours. From this, the conclusion has been drawn (Stephenson) that the metabolic activity in terms of cell material does not vary. Such a conclusion implies, however, that nitrogen content reflects quantity of cell material present or, in other words, that nitrogen forms a constant proportion of the cell. Oxygen uptake does, however, vary with respect to weight at different

stages of growth, being more vigorous in young cultures generally (see Meyrath & McIntosh, 1963b). Incorporation of nitrogen, being a metabolic activity, might be expected to rise and fall with other aspects of metabolic activity. The constant relationship between oxygen uptake and nitrogen content shown by Hershey & Bronfenbrenner (1938) may then be accounted for by postulating that during growth there is, in fact, fluctuation in oxygen uptake in relation to weight, but that it is paralleled by fluctuation in nitrogen assimilation. This seems to be more logical than concluding that oxygen uptake does not fluctuate during growth.

One of the main conclusions to be drawn from studies on carbon and nitrogen metabolism is that the cultures seem to undergo an aging process. A small-inoculum culture assumes the properties of an old large-inoculum culture on reaching the same mycelial content as a large-inoculum culture at a fairly early stage of growth. The aging of the mycelium is accompanied by a decrease in efficiency, so the result is that at comparable mycelium contents, the large-inoculum culture is usually the more efficient.

SECTION 4

RESPIRATION

4 RESPIRATION

41 INTRODUCTION

Earlier in this work, the following factors in relation to size of inoculum in Aspergillus oryzae have been among those considered: (a) carbon metabolism, (b) nitrogen metabolism and (c) effect of phosphates, trace elements and the products of the mould's own metabolism. The respiratory activities of the organism are considered below. Some aspects of these studies have already been considered in part in the appropriate section (e.g. CO_2 output with C metabolism). It was also shown earlier in this work (Fig.32, p.124) that the rate of uptake of oxygen was very similar in stationary cultures from large and small inocula. It is important to note, however, that such stationary cultures showed little effect of inoculum size in other respects, with the result that respiratory activities were in agreement with other findings. All the respiratory experiments that follow were performed on deep (vibrating stirrer) cultures, and it was shown earlier that effects of inoculum size commonly occurred in such cultures.

It was also shown in the preceding studies that the

properties of mycelium altered during growth of a culture. Substances which stimulated and substances which inhibited growth (mycelium formation) were excreted at various stages of culture development and the size of inoculum used was found to be a determining factor for the production of these. It was considered that the respiratory properties of the mycelium, at various stages of growth, might alter together with other properties and so act as an indicator of change in the properties of the mycelium. As the work proceeded, it was found necessary to examine not only the respiratory properties of the mycelium, but also the respiratory techniques themselves, paying particular attention to the treatment the mycelium received prior to testing. It followed too that as the culture was sensitive to changes in the composition of the substrate (McIntosh & Meyrath, 1964b), it might be profitable to study the effect of various suspending fluids on the respiratory behaviour of the mycelium. It was also of interest to know whether the substances shown to be responsible for stimulating and inhibiting growth would affect respiration.

Examination of all of these factors was necessary in order to establish a method which would give results indicating the respiration in the growing culture. Warburg

techniques were used throughout these experiments and although electrode methods for determination of specific uptake of oxygen being currently developed (see Hospodka, Čáslavský, Doran & Štros, 1964; Arthur, 1965) are likely to be of value in this kind of work, a number of problems still remain. Estimation of rate of uptake of oxygen in very dense cultures, for example, poses problems in both electrode and Warburg methods.

42 METHODS

421 Growth techniques

All respiration experiments described here were performed on the mycelium of deep (vibrating stirrer) cultures grown from large or small inocula. The method of growing these cultures was described earlier (p.38). Conidial inocula of Aspergillus oryzae were used throughout; these were prepared and inoculated into the substrate in the manner already described (pp.40,41). The substrate was A_4 10g/l starch (Table A, facing p.34) and the amplitude of the vibrating stirrer was adjusted to 1 mm.

The temperature of incubation was 25°. When each sample for a respiration experiment was withdrawn, the sample was made sufficiently large to permit a mycelial dry weight estimation (see pp.43,44) at the same time.

Deep cultures were chosen for respiration studies for the following reasons: (a) they provided amounts of mycelium which could be obtained from stationary flasks of very large surface area only; (b) the mycelium was uniformly distributed and not in a thick felt as with stationary cultures so that sampling could be more easily undertaken; (c) large samples could be taken at early stages when the mycelial content was small; (d) the uniformly distributed mycelium could be easily and effectively washed and distributed into small Warburg flasks.

At intermediate stages of growth the samples could be examined without further treatment. At other stages of growth, adjustment was necessary to give a suitable rate of exchange of gas for the particular apparatus available. For example at early stages of growth, the mycelium content of the culture was so small that the culture required to be concentrated in order to give a workable amount of mycelium; at late stages the culture required to be diluted before use. These adjustments necessarily resulted in preparations which

were not strictly comparable because of the various treatments involved. This fact required to be kept in mind when comparing samples at different stages of growth.

422. Respiration techniques

Uptake of oxygen and output of carbon dioxide were measured manometrically. Flasks for manometric measurement, ranging in capacity from 13 up to 23 ml, received 2 ml mycelial suspension containing about 3 mg dry weight mycelium. In the side arm was placed 0.5 ml of a 1% w/v solution of glucose unless otherwise indicated. When oxygen uptake was being estimated 0.2 ml of 20% KOH was placed in the centre well the inner rim of which was coated with lanolin to prevent creeping of the alkali. The manometers were shaken at 103 cycles per min with an amplitude of 4.2 cm. In other respects the procedure of Umbreit, Burris & Stauffer (1964) was followed.

The rate of carbon dioxide production was estimated by the direct method of Warburg. When the growth substrate served as suspending medium for the mycelium, the pH was determined and the solubility of carbon dioxide calculated

according to the formula

$$\alpha' = 0.759\ [\text{antilog}\ (\text{pH} - 6.34) + 1\ ];$$

α' indicates the solubility of carbon dioxide at the given pH.

The gas exchange reactions were usually followed in duplicate at 15 min intervals over 3 h. Zero order reactions were obtained over a period of 2 - 3 h; the induction phase was of short duration. Only the initial linear part of the curve has been taken into account in the estimation of the metabolic activity. This is expressed as µl gas exchanged in one hour at 0° and 760 mm Hg per mg mycelium dry weight (µl/mg h).

The mycelium was prepared in a variety of ways. In order to obtain what is commonly referred to as 'resting mycelium', the mycelium was usually collected on a fine mesh fabric of nylon or cellulose acetate spread over a Büchner funnel connected to a suction flask. The cell material was freed from adhering substrate by treatment in one of two ways: (a) by pressing firmly on the filter fabric in the Büchner funnel with a rubber bung three times, rinsing with the appropriate washing agent in between; (b) as before, followed by several firm pressings between filter paper, till no wetting of the paper was evident. The latter procedure was followed when the net weight of mycelium was determined.

43 FACTORS INFLUENCING THE RESPIRATION ACTIVITY

At an early stage of these investigations, difficulties were encountered in assessing the respiratory activity of the growing culture since slight variation in the preparatory procedure could result in considerable variation in the respiration rate of the sample. It was therefore considered of importance to discover and examine the factors influencing the respiratory activity in Warburg flasks. This would provide the basis for a more reliable assessment of the actual respiration rate in the growing culture.

431 Influence of treatment of mycelium

During the work on respiration, an interest in the treatment of the mycelium preparatory to examination by the Warburg techniques was aroused.

In examination of bacteria, it is common to use washed cells for respiration studies; washing is considered to remove most nutrients and to render cells "resting". The advantages of this procedure are listed by Umbreit, Burris & Stauffer (1964) who also state "the techniques applicable

to one type of tissue are not necessarily suitable for another" Preparation of samples from mould cultures tends to be less adequately described. Umbreit, Burris & Stauffer (1964) state "Both moulds and actinomyces normally grow as a heavy mat over the surface of media. This mat may be handled in much the same manner as animal tissues. It may be removed, washed with water and cut into slices" The same authors also point out the value of aerated submerged culture methods in producing mycelium in a form more easy to handle. Brief as is the description, it is to be noted that the treatment method recommended for moulds involves washing, presumably in an attempt to obtain resting cells.

Resting cells have been used widely in dissimilation studies to prevent growth during an investigation. An extension of their use to Warburg work was a logical consequence. In a warm shaking Warburg bath, cells might be expected to proliferate if in contact with nutrient solution, leading to increased enzyme formation and consequent lack of linearity in the curve for uptake of oxygen. It was determined nevertheless to make use of direct samples as being a more likely indication of the true nature of the growing culture, and to compare these with preparations in which the mycelium

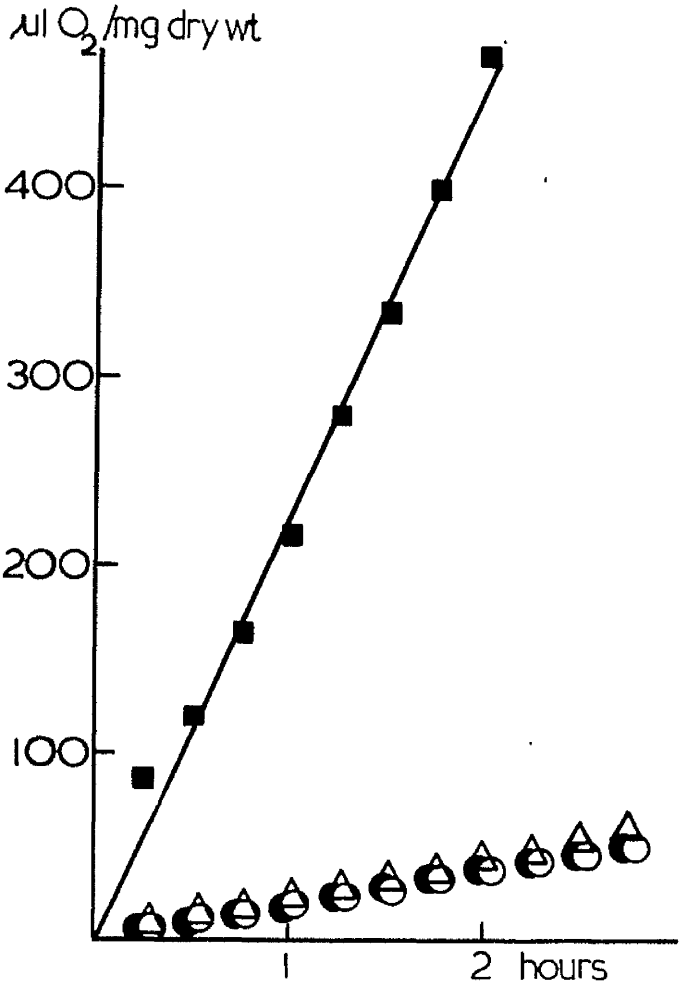


Fig.38 Effect of treatment of mycelium on uptake of O_2 in a deep culture of Aspergillus oryzae from a large inoculum. The culture was 30 h old and had a mycelial content of 6.8 mg/100 ml.

■ - ■ direct sample

● - ● washed with water, pressed, suspended in water

○ - ○ washed with water, pressed, suspended in phosphate buffer

△ - △ washed with phosphate buffer, pressed, suspended in same

The phosphate buffer was at concentration of substrate A4.

was treated in various ways. It was found in fact, that rate of uptake of oxygen in a direct sample was usually much higher than that in a treated sample. Moreover there was, in fact, no increase in rate of respiration since a linear relationship held over a considerable portion of the curve. The process of transference of the mycelium from the culture vessel to the Warburg flask seemed to produce a stasis in growth which was long enough to allow estimation of oxygen uptake rates.

It is generally true, then, that details of preparatory treatment go unrecorded in the literature, although awareness of the importance of such treatment has been shown (Hromatka & Ebner, 1949; Wikén & Somm, 1952). If the influence of the procedures used is known, it will assist in the interpretation of results (McIntosh & Meyrath, 1965).

Fig.38 shows the results of an experiment performed on a large-inoculum culture at an early stage of culture development (6.8 mg/100 ml). A direct sample showed a markedly higher rate of uptake of oxygen than did a similar sample which had been washed, pressed and re-suspended, using water or phosphate buffer for the washing or re-suspending. This is a preliminary indication of the marked effect that

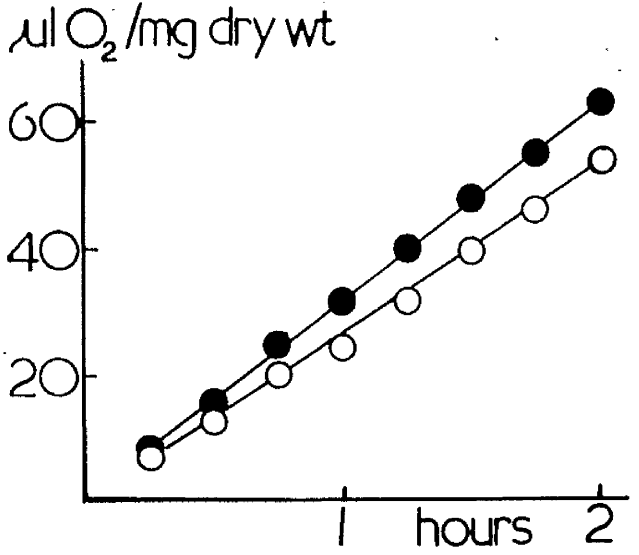


Fig. 39 Effect of pressing on rate of uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The culture was 34 h old and had a mycelial content of 31.9 mg / 100 ml.

● – ● Concentrated on filter: no pressing, no washing: suspended in filtrate

○ – ○ Pressed in presence of filtrate. Moistened with filtrate and pressed as usual (not with filter paper): suspended in filtrate

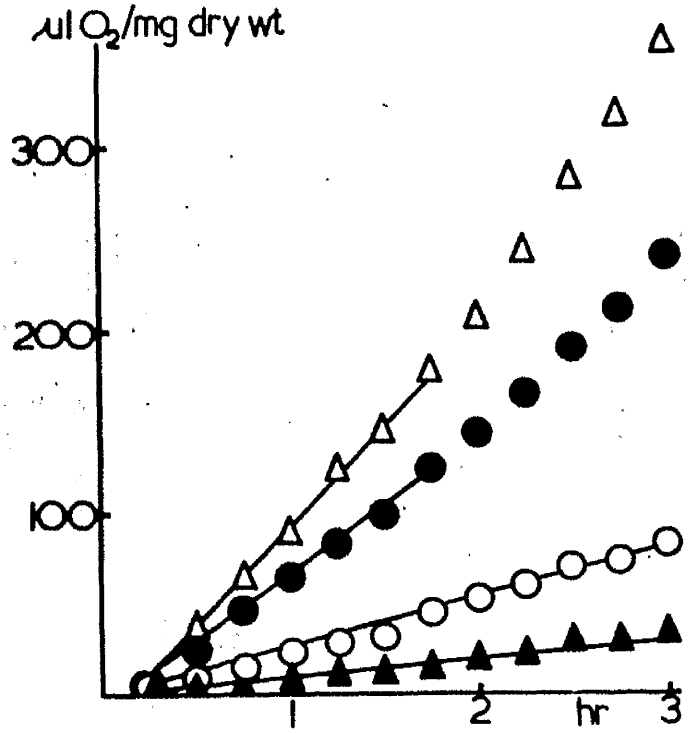


Fig. 40 Effect on uptake of O_2 of suspending agent for and treatment of mycelium from a small-inoculum deep culture of Aspergillus oryzae. The culture was 68 h old and had a mycelial content of 2.61 mg/100 ml.

◉ - ● concentrated on filter, without washing, suspended in filtrate

○ - ○ concentrated on filter, washed only with water, suspended in water

▲ - ▲ concentrated on filter, washed with water and pressed, suspended in water

△ - △ concentrated on filter, washed with water and suspended in a substrate with glucose as source of carbon

may be brought about by treatment of mycelium. In this case a dramatic reduction in uptake of oxygen of from 250 to 20 µl/mg h was obtained. Treatment in this instance included pressing and washing. These factors are considered separately below, as are other treatment factors such as filtration and storage.

4311 Effect of Pressing

Pressing of mycelium to free it from substrate is common practice. Fig.39 shows that the influence on rate of uptake of oxygen brought about by pressing the mycelium on the cellulose acetate filter is not marked. Fig.40 shows that while washing with water and suspending in water reduced the rate of uptake of oxygen considerably (○ - ○ compared with ● - ●), pressing as well (▲ - ▲) had only a little additional effect. Although suspension in a glucose-containing substrate led to an eventual departure from linearity in the curve, linearity was maintained for about two hours. Other work has shown that there is some complexity in the relationship between pressing and respiration, and that the topic would repay further study.

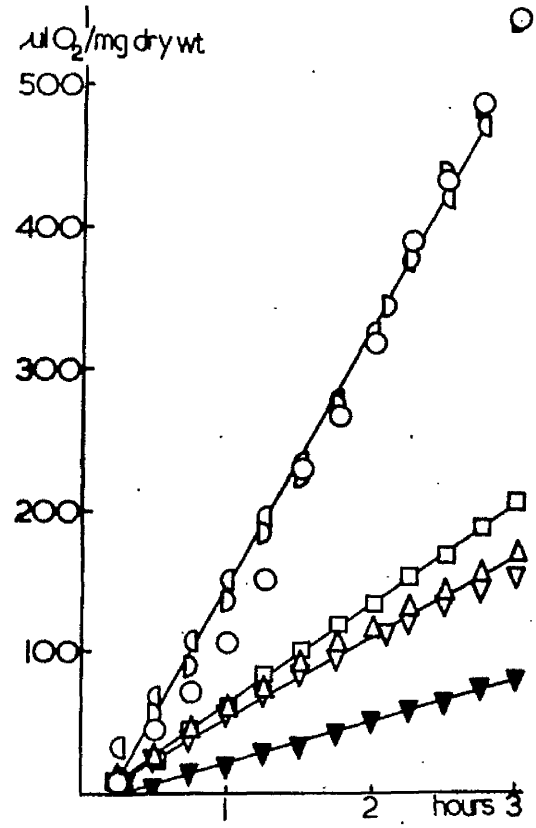


Fig. 41 Effect on uptake of O_2 of washing of mycelium from three different stages of growth of a large-inoculum deep culture of Aspergillus oryzae.

sample	h	mg/100 ml	treatment
1	23.5	5.6	○ - ○ direct △ - △ washed with ~ 100 ml water, suspended in water
2	28	13.6	◇ - ◇ direct ▽ - ▽ washed with ~ 100 ml water, suspended in water
3	31	28	□ - □ direct ■ - ■ washed with ~ 100 ml water, suspended in water ▼ - ▼ washed with ~ 800 ml water, suspended in water

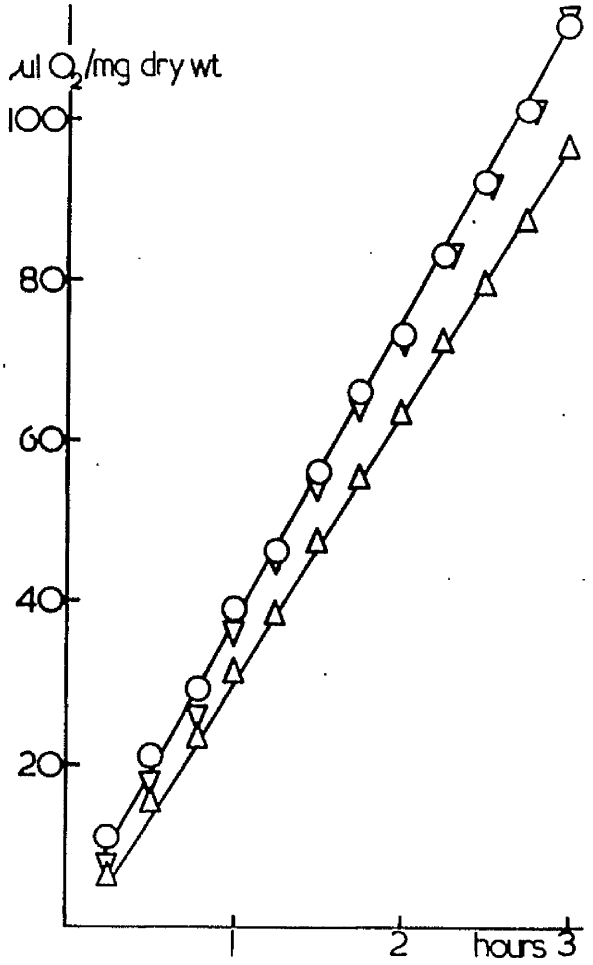


Fig.42 Effect on uptake of O_2 of washing of mycelium from a late stage of growth of a large-inoculum deep culture of Aspergillus oryzae. The culture was the same as that described in Fig.41 but the sample was taken after 48 h and had a mycelium content of 218 mg/100 ml.

○ - ○ direct sample

△ - △ washed with ~ 100 ml water, suspended in water

▽ - ▽ washed with ~ 800 ml water, suspended in water

4512 Effect of Washing

A more detailed investigation of the influence of washing and suspending in water, performed on a large-inoculum culture, is shown in Figs.41 and 42. Water was the only washing and suspending agent used except when direct samples were examined. Samples of culture at various times were taken and the effect of washing evaluated. The first three samples were taken from early stages of growth (23.5 h, 5.6 mg/100 ml; 28 h, 13.6 mg/100 ml; 31 h, 28 mg/100 ml) and the direct samples showed an almost identical rate of uptake of oxygen on each separate occasion (Fig.41). Washing in about 100 ml water gave a marked and almost identical reduction in rate of oxygen uptake in each case (Fig.41). The third of these samples was also examined by a more extensive washing process, using 750 ml instead of 100 ml water for washing. It can be seen that this procedure reduced still further the rate of uptake of oxygen. When mycelium from a fourth (late) stage of growth was examined (48 h, 218 mg/100 ml, Fig.42), the decrease in rate of oxygen uptake expected from earlier work took place. From the first to the fourth sample, there was found to be a decrease from 200 to 36 µl/mg h in the rate of oxygen uptake

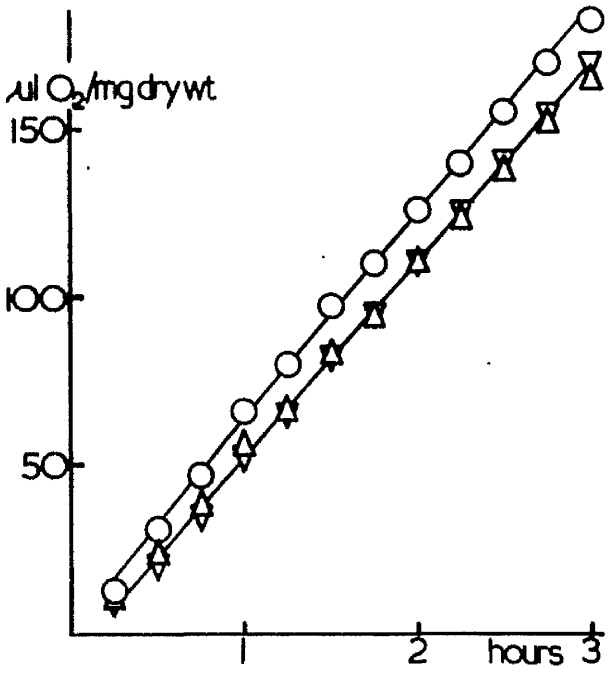


Fig. 43 Effect on uptake of O_2 of washing of mycelium from the stage of autolysis of a large-inoculum deep culture of Aspergillus oryzae. The culture was the same as that described in Figs. 41 and 42, but the sample was taken after 55 h and had a mycelium content of 139 mg/100 ml.

○ - ○ direct sample

△ - △ washed with ~ 100 ml water, suspended in water

▽ - ▽ washed with ~ 800 ml water, suspended in water

by the direct method. It was of interest to note however that washing by a small or large volume of water at a late stage reduced the rate of oxygen uptake hardly at all. This reduced sensitivity was also shown in the phase of autolysis (55 h, 139 mg/100 ml, Fig.43). At this stage, however, rate of uptake of oxygen was actually greater than at a slightly earlier stage, prior to autolysis (Fig.42); rate of uptake of oxygen increased from 36 to 62 µl/mg h. Meyrath (1965) has already shown that specific production of amylase increases during the phase of autolysis and that the latter cannot therefore be regarded as a time of invariable decrease in activity. The finding on rate of uptake of oxygen confirms this view.

4313 Effect of filter fabric and centrifugation

It was considered that the filter fabric might have some effect on the respiratory activity of mycelium which had been collected on it. The filter fabric which had been used was usually of either nylon or cellulose acetate. When the fabric was detergent-cleaned then given a thorough rinsing in tap and distilled water to ensure its cleanliness, some

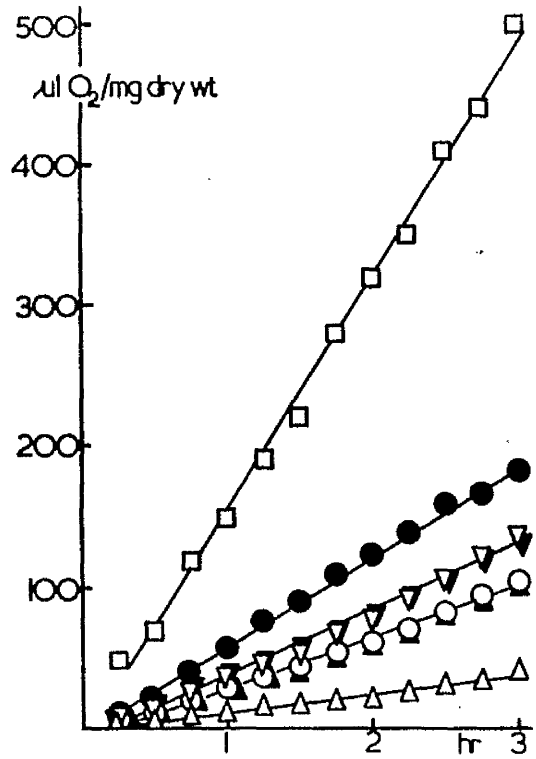


Fig.44 Effect on uptake of O_2 of various methods of treatment of mycelium from a large-inoculum deep culture of Aspergillus oryzae. The culture was 23.5 h old and had a mycelial content of 4.9 mg/100 ml at collection. It had been kept in the refrigerator for 6 h. The mycelium was collected on a detergent-treated filter.

□ - □ direct sample

△ - △ concentrated on filter without treatment, suspended in filtrate

○ - ○ washed with filtrate, suspended in same

▲ - △ washed with substrate, suspended in substrate

▽ - ▽ washed with water, suspended in filtrate

▽ - ▽ washed with water, suspended in substrate

◎ - ◎ washed with water, suspended in water

Note: cellulose acetate filter treated with detergent, rinsed in hot and cold tap water, rinsed in distilled water before start of experiment and after each sample treated.

interesting effects were obtained.

The mycelium chosen for this examination was from a large-inoculum culture at an early stage (25.5 h, 4.9 mg/100 ml) of growth. A direct sample gave the expected high rate of uptake of oxygen (Fig.44). When the mycelium was concentrated on a detergent-treated and washed filter without treatment (that is, neither washing nor pressing) and re-suspended in filtrate, there was a very strongly marked reduction in rate of uptake of oxygen (13 as compared with 160 µl/mg h). Since these conditions were meant to make the mycelium comparable to that in a direct sample in every respect except one, namely, contact with the filter, the effect must be due almost entirely to the filter. It has been suggested (Meyrath & McIntosh, 1964) that this marked decrease in rate of uptake of oxygen was due mainly to an adsorption of the mycelium on to the filter. It was also found that washing with filtrate or substrate and suspending in the same agent reversed this effect to some extent; washing with water and suspending in filtrate or substrate reversed it still more; washing with water and suspending in water reversed it most of all, although the rate of oxygen uptake did not revert to that of the direct sample (Fig.44). It should be noted that in this instance it made

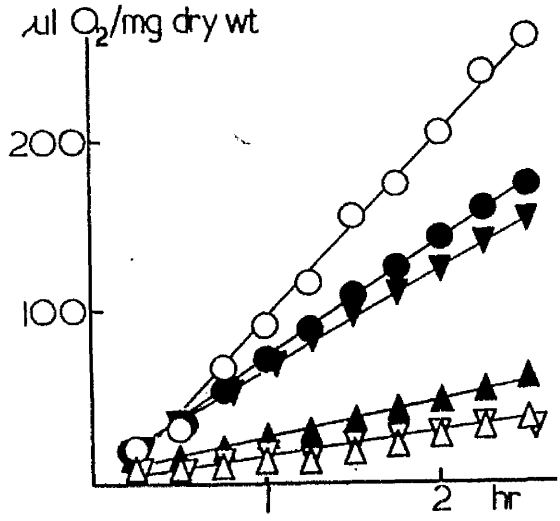


Fig.45 Effect on uptake of O_2 of means of collection (by filtration or centrifugation) of mycelium from a large-inoculum deep culture of Aspergillus oryzae. The sample was the same as that described in Fig.44 but after collection it had been kept in the refrigerator overnight. When filtration was the means of collection, a detergent-treated filter (see Fig.44) was used.

centrifuged
- ◉ - ◉ washed with water, suspended in water
- ○ - ○ washed with original substrate, suspended in original substrate
- ▽ - ▽ washed with water, suspended in supernatant

filtered
- △ - △ washed with water, suspended in water
- ▲ - ▲ washed with filtrate, suspended in filtrate
- ▽ - ▽ concentrated on filter, suspended in filtrate

no difference whether substrate or filtrate was chosen as suspending agent. The washing agent was of importance, however, and filtrate or substrate was more inhibitory than water.

In the preceding experiment results for a detergent-treated filter had been compared with those from a direct sample. It was considered that centrifugation might be attempted as a means of collecting mycelium as it allowed the collection of mycelium without recourse to a filter fabric intermediary. This was done together with a repeat of some of the filtration methods using mycelium taken from the same culture at the same time as that for the preceding experiment, but the mycelium had been kept overnight in the refrigerator (see Fig.45). The tests done on detergent-treated filter gave the expected very low rate of oxygen uptake after concentrating on the filter and suspending in filtrate. Although the rate after washing with water and suspending in water was lower than that obtained previously, the rate of oxygen uptake after washing with filtrate and suspending in filtrate was again almost as before. Centrifugation, however, was much less inhibitory by any method tried as can be seen from Fig.45, again emphasising the importance of the part played by the surface of the filter.

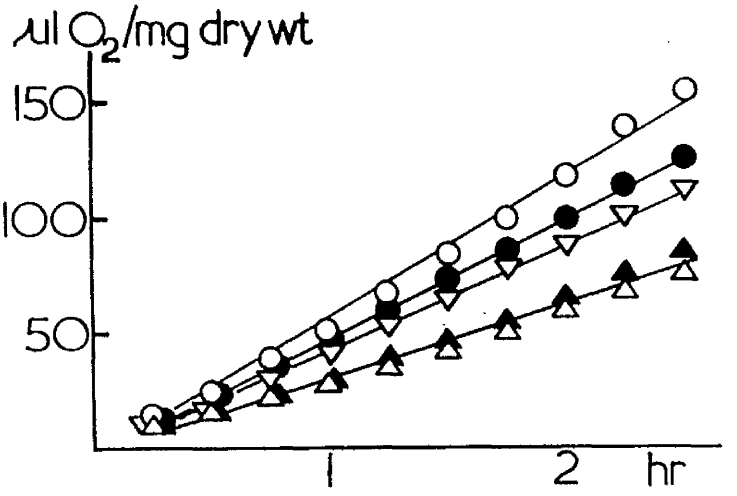


Fig.46 Effect on uptake of O_2 of means of collection (by filtration or centrifugation) of mycelium from a large-inoculum deep culture of Aspergillus oryzae. The culture was the same as that described in Figs.44 and 45 but the sample was 48.5 h old and had a mycelium content of 260 mg/100 ml. When filtration was the means of collection, a detergent-treated filter (see Fig.44) was used.

⊛ - ⊛ washed with water, suspended in water
O - O washed with substrate, suspended in substrate
} centrifuged

△ - △ washed with water, suspended in water
▲ - ▲ washed with substrate, suspended in substrate
▽ - ▽ washed with filtrate, suspended in filtrate
} filtered

The higher rate of respiration with mycelium washed by centrifuging as compared with washing on a filter which has not been treated with detergent (e.g. see Fig.41) may be due(in part) to residues of substrates left in the interstices of mycelium washed by centrifuging. It was difficult to separate the cell material effectively from its substrate because of the bulky nature and the low specific gravity of the mycelium.

Fig.46 again shows a comparison between a centrifugation method and a detergent-treated filter method. The same culture as is described in Figs.44 and 45 was chosen but the mycelium was from a late stage of growth (48.5 h, 260 mg/100 ml). Fig.46 shows that a difference in rate of oxygen uptake between centrifuged preparations and those collected on detergent-treated filters was not so readily distinguishable as in the preceding experiment (Fig.45). The curves for both procedures tend to merge instead. This may be explained by the fact that, being at a late stage, the content of mycelium of the culture was fairly large; contact of such mycelium with the filter will be correspondingly less and so the effect of adsorption by the filter will not be so apparent. Moreover, from previous work, the mycelium would be expected to be less sensitive at a later stage of growth.

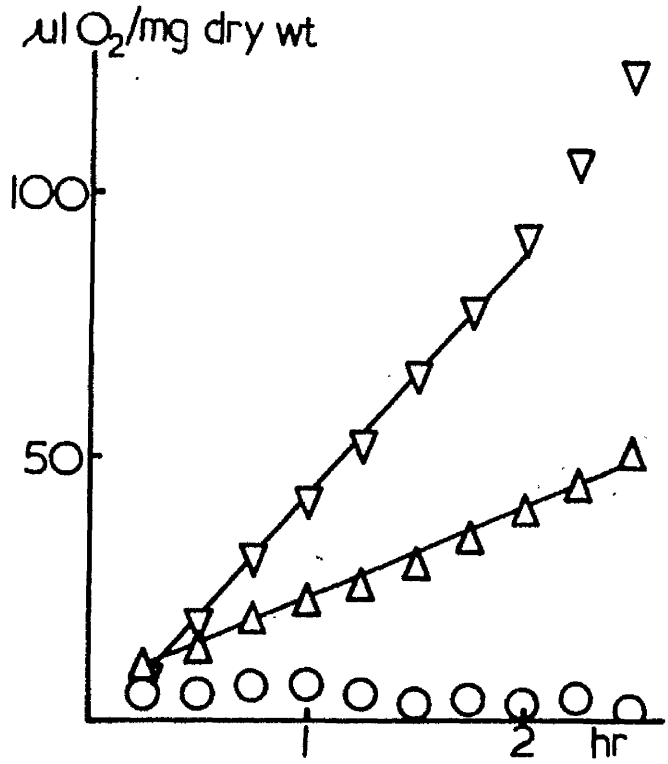


Fig.47 Effect of suspending medium on uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The culture was 29 h old and had a mycelial content of 1.86 mg/100 ml.

O - O washed only, not pressed, suspended in water

△ - △ washed only, not pressed, suspended in filtrate

▽ - ▽ washed only, not pressed, suspended in original substrate

4314 Effect of Storage

It was considered useful to determine whether refrigeration and the accompanying contact over a period of time with various suspending fluids were responsible for any change in the rate of uptake of oxygen. Figs.47, 48 and 49 show the results of a series of experiments devised to determine the influence of refrigeration on rate of uptake of oxygen. Mycelium from a large-inoculum culture at an early stage of growth (29 h, 1.86 mg/100 ml) was used. The samples were thoroughly washed in water, but not pressed, before suspending. Water, filtrate or substrate was the suspending agent used. With mycelium from this stage, and using fresh samples (Fig.47) the rate of oxygen uptake when the mycelium was suspended in original substrate was 52 µl/mg h. On suspending in water, there was almost no uptake of oxygen. Suspending in filtrate gave an intermediate result. These results show clearly that substrate was stimulatory to rate of oxygen uptake as compared with water, presumably due to one or more of the constituents of the substrate. The fact that filtrate was less stimulatory suggests either that inhibitory substances were excreted during growth or that some of the stimulatory substances of the original substrate were used up during growth.

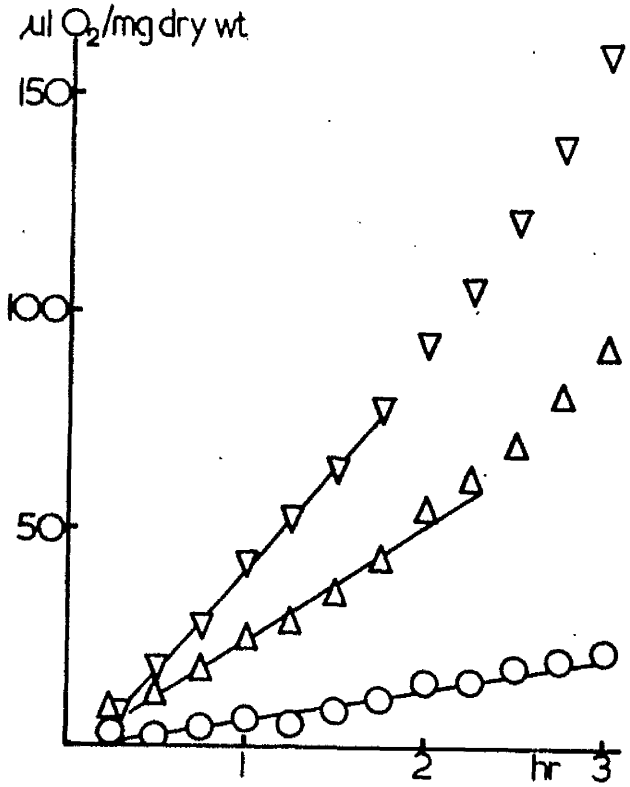


Fig.48 Effect of extended contact with suspending medium on uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The mycelium samples were those of Fig.47 which had been stored in their respective environments at 2° for 16 h before re-testing, i.e. the following treatment had been performed 16 h before testing.

○ - ○ washed only, not pressed, suspended in water

△ - △ washed only, not pressed, suspended in filtrate

▽ - ▽ washed only, not pressed, suspended in original substrate

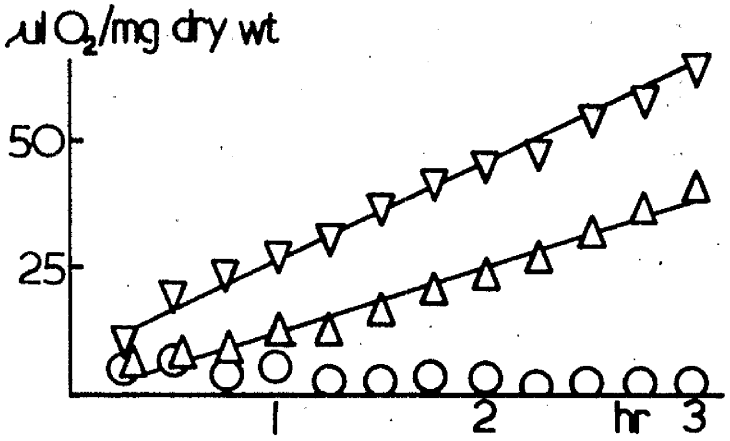


Fig.49 Effect of prolonged contact with suspending medium on uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The mycelium samples were those of Fig.47 which had been stored in their respective environments at 2° for 4 days before re-testing, i.e. the following treatment was performed 4 days before testing.

○ - ○ washed only, suspended in water

△ - △ washed only, suspended in filtrate

▽ - ▽ washed only, suspended in original substrate

When the mycelium in each case was stored in the various suspending fluids overnight (16 h) and re-tested without further treatment, a very similar pattern of oxygen uptake was obtained (Fig.48); overnight storage under refrigeration conditions was thus shown to make very little difference to the effect obtained.

When this period of contact with suspending fluid and storage under refrigeration was extended over four days, the relationship of one method to the other still held good although there was an all-round decrease in rate of uptake of oxygen (Fig.49).

432 Influence of substrate constituents

In the foregoing work on the influence of treatment of mycelium, variants had been introduced which showed that it was important whether water, original substrate or filtrate was used in making the preparation. It was often of interest to try to reproduce the conditions of the actual culture, even where, for some reason, a direct sample could not be used. It is reasonable to conclude that a direct sample was most closely simulated when filtrate was used as

suspending agent. Filtrate differed from original (unfermented) substrate in that the former had been subjected to depletion or supplementation due to the biochemical activities of the mould. Original substrate might be expected to have an effect which depended upon the nature and balance of its constituent substances.

It was also borne in mind that although starch was the carbohydrate provided in the substrate, 0.5 ml of 1% glucose was added from the side arm of the manometric flask when testing for respiratory activity (see Methods p.138). On each occasion of adding an untreated sample from a culture, some carbon source was thereby introduced to the flask. Since the concentration of carbon source changed during cultivation, a variable amount of carbon source was being added. It was considered important to know whether this alteration in concentration of carbon source affected the rate of uptake of oxygen, particularly when it had been shown in Fig.40 (p.143) that washing in water and suspending in a glucose substrate was stimulatory in comparison with omitting washing then suspending in filtrate. The influence of such factors is considered at this point.

To determine whether the concentration of glucose normally added was important, the respiration activity obtained when 1% glucose was used was compared with that

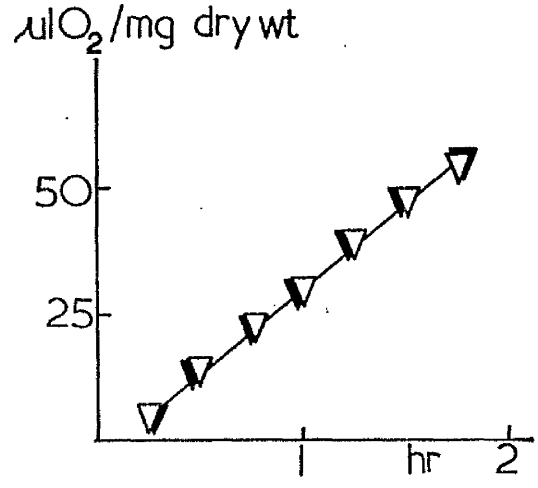


Fig. 50 Effect of concentration of the glucose solution added to the reaction flask on testing the uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The culture was 34 h old and had a mycelial content of 31.9 mg/100 ml.

▽ - ▽ 0.5 ml 1% glucose added per flask

▽ - ▽ 0.5 ml 10% glucose added per flask

The mycelium had been washed with water, suspended in water and kept in the fridge for about 5 h.

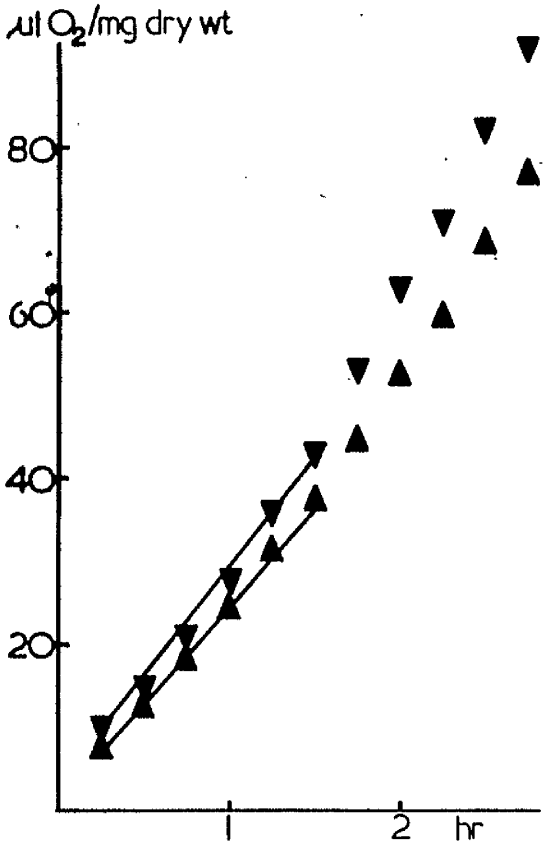


Fig.51 Effect of suspension in substrate or filtrate on uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The culture was 30 h old and had a mycelial content of 6.8 mg/100 ml. This experiment was performed on the same sample of culture as that described in Fig.38.

▲ - ▲ washed with water, pressed, suspended in original substrate

▼ - ▼ washed with water, pressed, suspended in filtrate

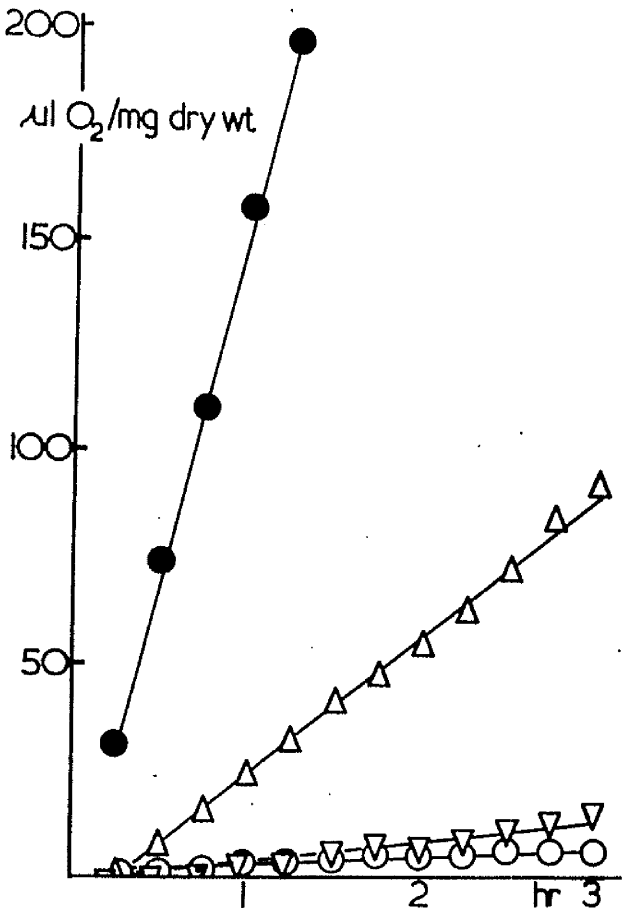


Fig.52 Effect on uptake of O_2 of treatment of mycelium from a large-inoculum deep culture of Aspergillus oryzae. The growth curve for this culture is given in Fig.7 (closed circles)

sample	h	mg/100 ml	treatment
2	24.5	16.6	◎ - ◉ direct
8	191	52	△ - △ direct
			○ - ○ washed, pressed, suspended in water
			▽ - ▽ washed, pressed, suspended in filtrate

given when 10% glucose was used. Fig.50 shows the rate of uptake of oxygen to be almost identical in each case, so it was concluded that the concentration of added glucose was not critical within these fairly wide limits.

Mycelium treated as described in Fig.38 and originating from the same culture was examined for uptake of oxygen when the suspending fluid was filtrate (left after removal of mycelium) or original substrate, instead of water or phosphate buffer (Fig.51). This figure shows that the substitution of filtrate or substrate on this occasion led to a little growth after about 2 h, since from that time the curves are no longer linear. Over the initial, nearly linear part of the curve, however, it can be seen that at this stage there is little difference in rate of uptake of oxygen when filtrate is substituted for original substrate as suspending agent.

Mycelium from the phase of autolysis (191 h, 52 mg/100 ml) of a large-inoculum culture was also examined. At this late stage, the rate of oxygen uptake of the direct sample was again low when compared with that for an early stage sample (Fig.52). This rate was again greatly reduced by washing, pressing and re-suspending in either filtrate or water.

A comparison was made of the rates of oxygen uptake in

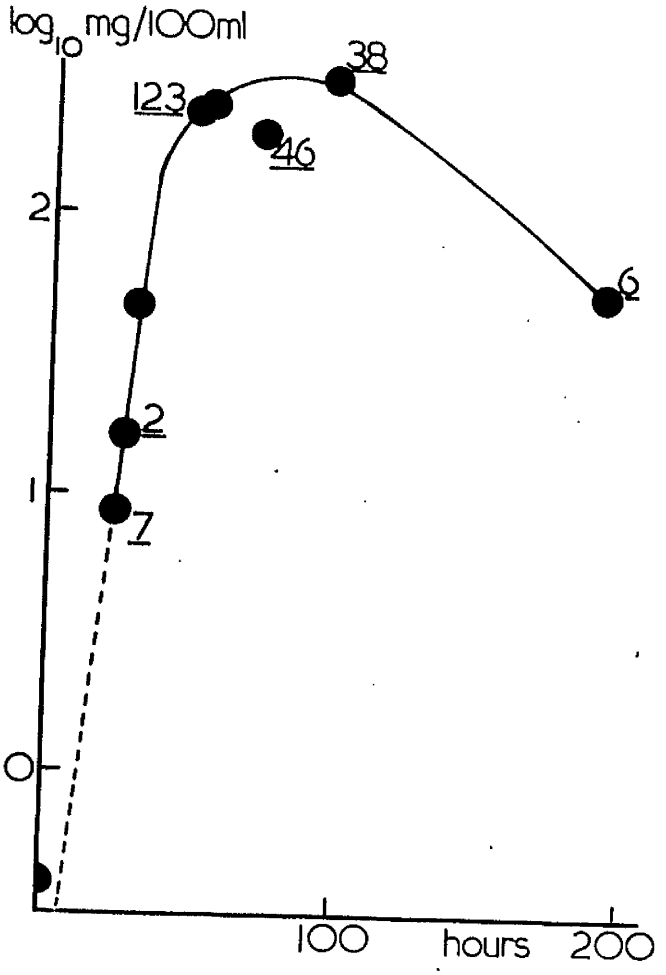


Fig. 53 The ratio

$$\frac{\text{rate of uptake of } O_2 \text{ in water}}{\text{rate of uptake of } O_2 \text{ in filtrate}} \times 100$$

shown (underlined) at various times in a semi-logarithmic plot of the growth curve of a deep culture of Aspergillus oryzae from a large inoculum. The culture was the same as that described in Fig. 52.

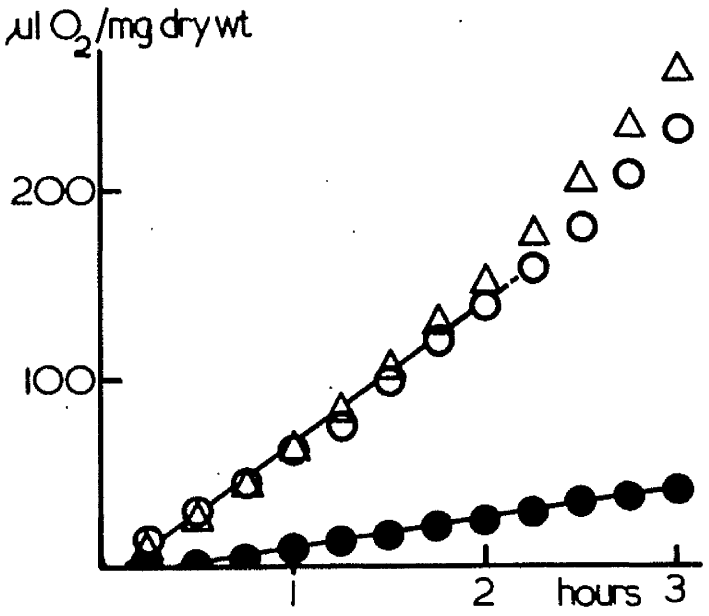


Fig. 54 Effect of treatment of mycelium on uptake of O_2 in a deep culture of Aspergillus oryzae from a large inoculum. The culture was 23 h old and had a mycelial content of 1.6 mg/100 ml.

● - ⊙ washed with water without pressing and suspended in water

○ - ○ washed with water without pressing and suspended in original substrate

△ - △ washed with water without pressing and suspended in filtrate

water and in filtrate. This was done by means of the following formula:

$$\frac{\text{rate of uptake of oxygen in water}}{\text{rate of uptake of oxygen in filtrate}} \times 100$$

The values for this ratio were calculated for various stages of growth and these have been entered in the growth curve on a semi-logarithmic plot (Fig.53). A value less than 100 means that water is more inhibitory than filtrate; the lower the value, the more inhibitory is water as compared with filtrate. It can be seen that in the exponential phase and the autolytic phase, water is markedly inhibitory, but that during intermediate phases it is much less so.

In Figs.51 and 52, recourse had been made to pressing procedures. It was decided that further examination of the influence of the suspending fluids should be made without using any pressing procedure whatsoever. Fig.54 shows the relationship in rate of oxygen uptake between water, original substrate and filtrate as suspending agents when the mycelium had been subjected to a process of washing without pressing. Again it should be noticed that there was evidence of very slight growth when filtrate or original substrate were provided as suspending fluid, as was shown by the curve being non-linear after about 2 h. No sign of such growth

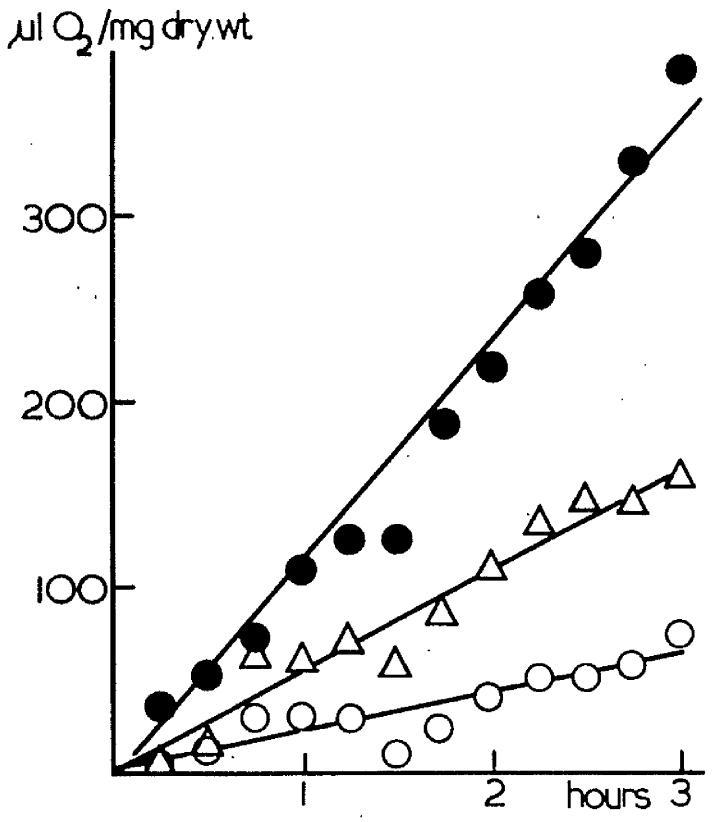


Fig.55 Effect of treatment of mycelium on uptake of O_2 in a deep culture of Aspergillus oryzae from a large inoculum. The culture was 31 h old and had a mycelial content of 5 mg/100 ml when collected. It had been kept in the fridge overnight. Treatment was after storage.

● - ● direct sample

△ - △ sample washed only and suspended in filtrate at the original concentration

○ - ○ sample washed only and suspended in water at the original concentration

was shown when the mycelium was suspended in water. From a comparison of the linear parts of the curves it can be seen (Fig.54) that filtrate and substrate as suspending agents were stimulatory to uptake of oxygen as compared with water, and that they were almost equally so. This was shown with mycelium from a large-inoculum culture at an early stage of growth (23 h, 1.6 mg/100 ml).

Another example of the stimulatory effect of filtrate as suspending agent in samples prepared without pressing is shown in Fig.55. The culture was from a large inoculum at an early stage of growth (31 h, 5 mg/100 ml). The portion of the culture from which the samples had been prepared had been kept in the refrigerator overnight. A direct sample was included on this occasion and it can be seen (Fig.55) that when pressing procedures were omitted, water was again inhibitory as compared with filtrate. Although pressing was omitted, mycelium which had been collected and re-suspended in its own filtrate was inferior to mycelium of a direct sample in rate of uptake of oxygen. This was probably due to a leaching out from the mycelium, during overnight storage, of materials which were not fully replenished by suspension in filtrate.

Fig.56 shows the influence of individual components of the substrate on respiratory activity. A large-inoculum

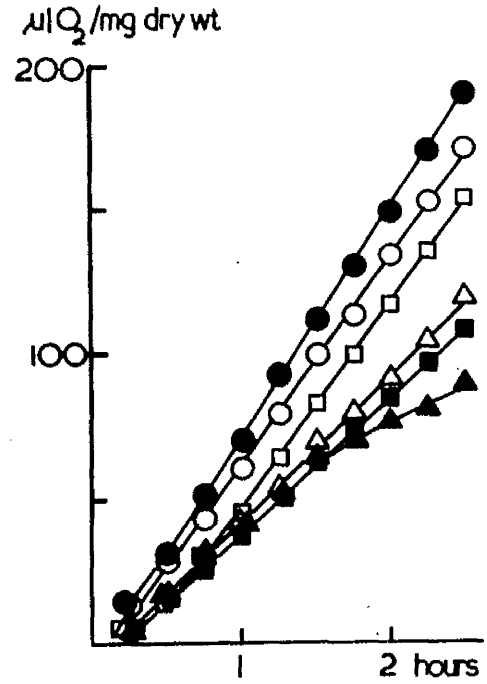


Fig.56 Effect of individual components of the substrate on uptake of O_2 by mycelium from a large-inoculum deep culture of Aspergillus oryzae. The sample was 24 h old and had a mycelial content of 6.1 mg/100 ml. The conidia on this occasion had been grown at 30°.

suspending agent

▲ - △ water

▣ - ▣ 3 parts trace element solution including Mg, 1 part water

△ - △ 3 parts $(NH_4)_2SO_4$ solution, 1 part water

□ - □ 3 parts trace element solution including Mg and $(NH_4)_2SO_4$, 1 part water

○ - ○ 3 parts substrate, 1 part water

◎ - ◎ 3 parts filtrate, 1 part water

culture at an early stage (24 h, 6.1 mg/100 ml) was examined. This experiment showed a very orderly relationship in the influence of the components within the substrate. Water gave a very low, if not the lowest, rate of uptake of oxygen. Trace element solution with magnesium showed a slight stimulation and, separately, did ammonium sulphate. Combined, these had a cumulative effect so that there was a greater stimulation than with either separately. The complete substrate was still more stimulatory and the filtrate even more so. This shows that we may expect each individual component of a substrate to exert a small effect and these effects may be cumulative. In the case of filtrate there is the added complexity of the presence of other products of the metabolism of the organism which may have a stimulatory or inhibitory effect on growth.

433 Influence of stage of growth

It has already been shown (section 2) that the properties of cultures from large and small inocula depended upon the stage of growth of the cultures. It was considered

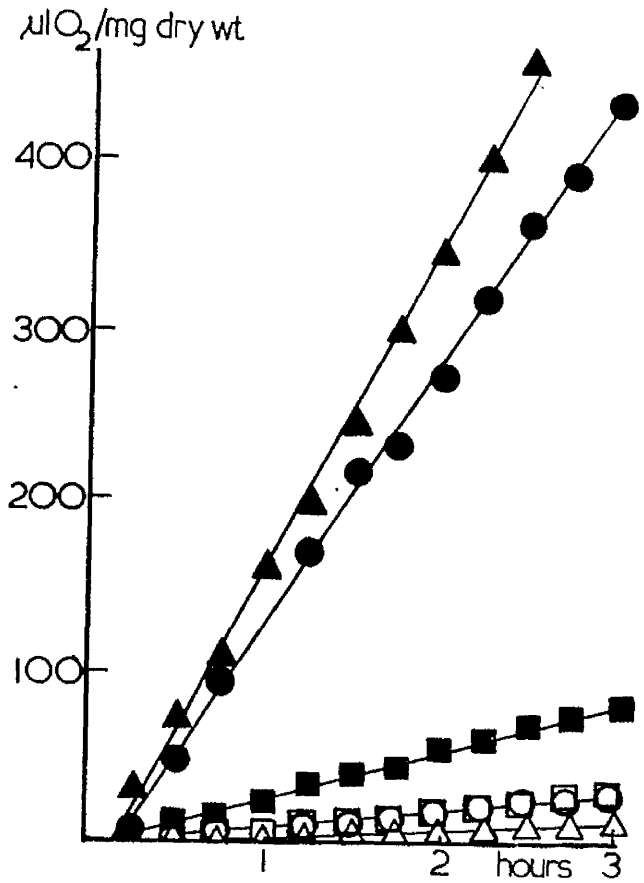


Fig.57 Influence of stage of growth and treatment of mycelium on uptake of O_2 in a deep culture of Aspergillus oryzae from a large inoculum. The growth curve for this experiment is given in Fig.7 (closed circles).

sample	h	mg/100 ml	treatment
1	21.5	8.7	●-● direct sample
			○-○ washed, pressed, suspended in water
2	24.5	16.6	▲-▲ direct sample
			△-△ washed, pressed, suspended in water
7	96	311	■-■ direct sample mixed with water
			□-□ washed, pressed, suspended in water

The culture was the same as that described in Figs.52 and 53.

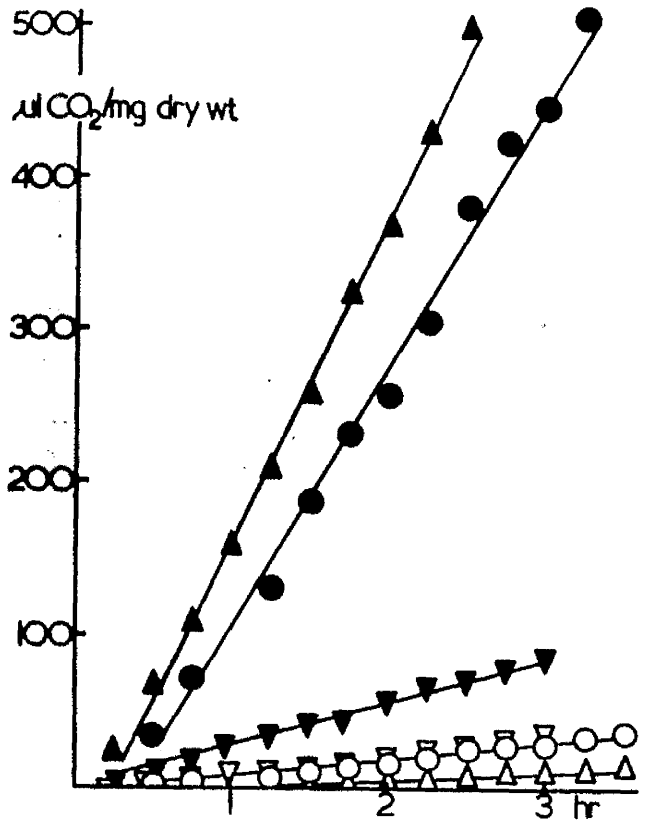


Fig.58 Influence of stage of growth and treatment of mycelium on output of CO_2 in a deep culture of Aspergillus oryzae from a large inoculum. The culture is the same as that described in Fig.57, and the methods of treatment also correspond.

sample	h	mg/100 ml	treatment
1	21.5	8.7	◎ - ◎ direct sample ○ - ○ washed, pressed, suspended in water
2	24.5	16.6	△ - △ direct sample △ - △ washed, pressed, suspended in water
7	96	311	▽ - ▽ direct sample mixed with water ▽ - ▽ washed, pressed, suspended in water

important to be able to show whether respiration activity varied with the stage of growth, that is, whether changes in behaviour of the mycelium was reflected in a change in respiratory behaviour. It was borne in mind that factors already considered, such as method of treatment of the mycelium and the influence of substrate constituents, would probably exert their own influence at various stages of growth.

In Fig.57, the difference in the rate of uptake of oxygen at various stages of growth of a large-inoculum culture of Aspergillus oryzae is shown. At early stages (21.5 h and 24.5 h), when the mycelial content of the culture was still low, the rate of uptake of oxygen of the mycelium in direct samples of the culture was high (150 and 190 µl/mg h respectively). At a late stage (96 h, 311 mg/100 ml) a direct sample showed the much reduced rate of uptake of oxygen of 30 µl/mg h (Fig.57).

Fig.58 shows that there was commensurate behaviour with regard to output of CO_2 in the same culture at the same stages of growth as in Fig.57. At the same two early stages examined, output of CO_2 was 170 and 195 µl/mg h respectively; at the corresponding late stage, the value was 30 µl/mg h. In both uptake of O_2 and output of CO_2,

then, there is a high rate at comparatively early stages of growth and a low rate at a late stage in large-inoculum cultures.

As well as showing a change in respiratory activity, Figs.57 and 58 show a change in the sensitivity of the mycelium during growth. Mycelium at all stages of growth was sensitive to the treatment used to provide 'resting' mycelium and such treatment decreased its rates of uptake of oxygen and output of CO_2 to values much lower than those for any direct sample. The rates were reduced to an almost uniform level, but the figures show that the sample with the highest rates by direct examination (sample 2) had the lowest rates after treatment as described, and the sample with the lowest rates by direct sample (sample 7) had one of the highest rates after treatment. These observations suggest that the mycelium is more sensitive to treatment at early stages than at late stages of growth in a large-inoculum culture and are in agreement with the results reported in Figs.41 and 42. In Table B (facing p.122) the results for oxygen uptake and carbon dioxide output have been combined to give the respiratory quotient at various stages of growth. The increase in Respiratory Quotient which may take place during growth of a culture (Table B) indicates that anaerobic dissimilation

(anoxybiontic fermentation, according to Bernhauer) takes place in older cultures.

The significance of this observation for studies on growth from large and small inocula becomes apparent when the respiratory activities of such cultures are compared at various stages of growth. At this point Fig.1 of Moyrath and McIntosh (1963b) should be consulted. This figure and the results already described not only show that the rate of uptake of oxygen was higher with young cultures than with old cultures but also show that this phenomenon occurs within the phase of exponential growth (Moyrath & McIntosh, 1963b). Moreover, cultures from large inocula during unrestricted growth at early stages showed a high specific rate of oxygen uptake (qO_2 = 100 - 220 µl/mg h), whereas older cultures were characterized by a much lower value (30 - 56 µl/mg h). Small-inoculum cultures grown under the same conditions gave rise to mycelium which showed much lower rates of specific oxygen uptake (qO_2 = 55 and 57 µl/mg h) at all stages of measurable growth.

4.4 DISCUSSION

Results obtained had been informative as to the influence of treatment methods, substrate constituents and stage of growth on respiration.

It was found that the usual pressing procedure could result in an only slightly diminished rate of uptake of oxygen. This was of interest in that it suggested that reasons for variation in respiration should be sought elsewhere. Refrigeration of cultures before testing also made little difference to their respiratory behaviour.

The filter fabric was found to play an important part in respiration studies. Fabric which had been detergent-cleaned reduced the rate of oxygen uptake strongly. It seems likely that there is a strong adsorption on to the filter accompanied by a removal of surface-located substances (Meyrath & McIntosh, 1964). Recent work has shown that the cell wall (as distinct from the cytoplasmic membrane) can act as a 'primary reservoir' for vitamin B_{12} in a B_{12}-requiring Lactobacillus (Suzaki & Kitahara, 1963). These observations indicate that the cell wall proper is not to be regarded as inert biochemically. Similarly, the work described in this section concerning the effect of

the filter fabric on the rate of respiration suggests that the phenomenon is associated with the characteristics of the cell wall. When a detergent-cleaned fabric was used, filtrate or substrate was more inhibitory than water as washing agent, suggesting that adsorption may not be so pronounced in water as in substrate.

The fact that the filter in particular was involved has been confirmed by substituting centrifuging for filtering as a washing procedure; it was not so inhibitory to rate of uptake of oxygen. Moreover, the difference between these two methods was much less marked with old mycelium which is to say that removal of these surface-active substances was with greater effect on rate of uptake of oxygen when it was done at an early stage of growth.

The washing and suspending agent used for the mycelium was shown to be of importance. Washing with water diminished the rate of uptake of oxygen. The more thorough the washing, the greater the reduction in rate that resulted. It seemed that substances were being removed by washing, because when mycelium was suspended in substrate it gave a better rate of uptake of oxygen than when suspended in water. When the individual components of the substrate were examined, they were found to be mostly stimulatory. The combined action of

ammonium sulphate and heavy-metal salts played an important part in this stimulation. It was considered, then, that the increased rate of uptake of oxygen might have been due to a stimulation of enzyme activity by metals which could be transported through the cell membrane by formation of a complex with ammonium ions to replace the metals leached by washing (Meyrath & McIntosh, 1963a). Old mycelium was not nearly so readily leached by washing.

These results are in agreement with earlier observations (Meyrath 1962a, 1963) that growth-stimulating or growth-inhibiting substances excreted by the mould required to act at early stages of growth to have an effect on late stages.

The effect of filtrate is more complex. Sometimes it was found stimulatory as compared with substrate, sometimes inhibitory. Filtrate may be expected to have an influence which is the resultant of a number of factors, namely the part utilization of stimulatory substances in the substrate and the production of self-stimulatory and self-inhibitory substances by the mould. The filtrate might then be expected to be either stimulatory or inhibitory as compared with substrate, depending upon the combination of factors concerned. This was found to be so.

The stage of growth was shown to be important in respiration studies, confirming in the case of Aspergillus oryzae what had been reported by other workers studying different organisms, namely, that the rate of uptake of oxygen was higher with young cultures than with old cultures (Ramiya, 1942; Hockenhull, Fantes, Herbert & Whitehead, 1954). This was plainly evident in a large-inoculum culture. These observations have been extended to show that this phenomenon occurred within the phase of exponential growth (Meyrath & McIntosh, 1963b). Additionally, it has been shown that the lower rate of uptake of oxygen by mycelium from a late stage of growth (large-inoculum culture) was accompanied by a decrease in the sensitivity of the mycelium to the various methods of treatment to which the mycelium was subjected.

The results indicate that mycelium from small-inoculum cultures at all stages of measurable growth behaved like mycelium from large-inoculum cultures at late stages of growth. It appears that small-inoculum cultures undergo a process of aging during the time they take to reach the same content of mycelium as in large-inoculum cultures at early stages of growth. If, as has been shown, the specific rate of uptake of oxygen decreases while the

rate of multiplication remains constant, it means that in later stages of growth less oxygen is required to form a given weight of mycelium. If the amount of energy required for synthesis of unit weight of mycelium is constant during exponential growth, and if a given amount of oxygen taken up releases a proportional amount of energy for cellular synthesis, then it might be assumed that in advanced stages of growth the required energy is made up by anaerobic processes, a view already put forward by Meyrath (1962b) in connection with anaerobic spoilage of fruit juices by moulds.

The work described above has shown that when respiration studies are performed on mycelium subjected to a variety of treatments, there are many reasons for viewing the results with discrimination. Probably the direct sample gives the best indication of the respiratory activity in the growing culture. The work has also laid a foundation for more extensive studies concerning the relationship of size of inoculum to respiratory activity.

SUMMARY

Reports by a number of authors in widely differing fields of biological study have shown that various phenomena accompany a change in the size of an inoculum. Such phenomena have been shown in the present work to occur in cultures of Aspergillus oryzae under a variety of conditions. The importance of size of inoculum in growth, in metabolism of carbon and nitrogen and in respiration has been considered. The size of the inoculum chosen has been found to be a determinative factor in each one of the aspects of metabolism studied.

It has been shown in growth experiments, particularly with reference to maximum yield that deep cultures are influenced by inoculum size to a greater extent than are stationary cultures and that increase in mechanical stress enhances the effect of inoculum size. Large-inoculum cultures usually show a higher maximum yield than do small-inoculum cultures.

The effects of inoculum size on growth are generally more pronounced when no trace elements are added. The effect of trace elements has been examined not only by using trace-element-poor chemicals, but by blocking trace

elements with a chelating agent, removing them by a purification procedure or adding them individually or in combination. It has been shown that each individual trace element makes its own contribution to the inoculum-size effect and the resultant effect is a composite one depending upon the balance of trace elements in the substrate. A balance can be struck which can give a reversal of the usual effects of inoculum size, that is, smaller growth rate or maximum yield are obtained with large-inoculum cultures. Caramelization of the substrate results in a masking of the effects.

Aspergillus oryzae has been shown to produce substances inhibitory and stimulatory to its own growth. Stimulating substances are evident in large-inoculum cultures at all stages of growth and in small-inoculum cultures at late stages of growth. Inhibitory substances predominate in early stages of growth of small-inoculum cultures.

Since the differences between results for the growth experiments were sometimes small, statistical methods were applied to assess the validity of claims made.

The metabolism of carbon and nitrogen has been studied in this work. Consideration of the utilization of the carbon source and production of acids, together with

incorporation and excretion of nitrogen has shown that large-inoculum cultures are generally more efficient regarding assimilation than are small-inoculum cultures at comparable mycelium contents. As the mycelium ages, it decreases in efficiency.

A number of factors influence the respiratory activity of Aspergillus oryzae. At late stages of growth, uptake of oxygen is reduced. Uptake of oxygen is reduced when the mycelium ages, or is subjected to a washing treatment, or is collected on a detergent-treated filter. Each individual component of the substrate has some influence on rate of uptake of oxygen, a combination of ammonium sulphate and trace elements being particularly effective. Small-inoculum cultures at all stages of growth capable of being collected and examined had the low rate of uptake found in late (but not in early) stages of growth of large-inoculum cultures.

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ACKNOWLEDGEMENTS

The author wishes to make grateful acknowledgement of all assistance accorded to him in this work. Thanks are due especially to:

.....Professor E.O.Morris, supervisor of the work, for his encouragement and advice

.....Dr.J.Meyrath, for general direction and day-to-day help and guidance, and for imparting some of his own enthusiasm for research work

.....Mr.J.McIlroy A.I.M.L.T., for the nitrogen estimations of section 3, help with the photography and excellent technical assistance throughout

.....the staff of the Department of Applied Microbiology and Biology and of the Microbiology section in particular, for willing help at all times

.....Dr.A.J.Lowie, Department of Mathematics, for advice on statistics, and that department for programming and processing some computer work

.....his wife, for typing drafts and final submission