THE EVALUATION OF THE FUNGI USING TOWER FERMENTERS

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Declaration

I hereby declare that the whole of the work now submitted in this thesis is the result of my own investigations except where reference is made to published literature and where assistance is acknowledged.

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Certificate

I hereby certify that the work embodied in this thesis has not already been submitted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

Signed B. Daunter.....
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Date ..20th March 1972.....

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This work is dedicated to the Father of Science, whoever he may be.

Summary

This investigation forms a part of a programme of work for the utilization of the carob bean Ceratonia siliqua by fungi for protein production. The work presented here is the development of a screening technique for the selection of suitable fungi for protein production utilizing an aqueous extract of the carob bean Ceratonia siliqua. A total of 37 fungi (19 Basidiomycetes and 18 Fungi Imperfecti) were surface cultured on an aqueous carob extract solidified with agar and supplemented with (i) Glutamic acid, (ii) Ammonium sulphate, (iii) Glutamic acid plus Ammonium sulphate, (iv) unsupplemented. The fungi were first cultured on malt extract solidified with agar, and 8mm diameter transplants were made from these cultures and transferred to the supplemented and unsupplemented carob extract. The rate of radial growth of these fungi were then measured. The fungi with the greater rates of growth were selected for screening in submerged culture. Statistical analysis as well as observations of colony growth were made in the selection of nitrogen supplements to be used in the screening of the fungi in submerged culture.

Of these 37 fungi, 6 Basidiomycetes and 5 Fungi Imperfecti were selected for screening in submerged culture in 1.0L tower fermenters; the media being of a similar composition to that used in the surface culture. From these 11 fungi screened in submerged culture, 3 Fungi Imperfecti were selected as possible dietary protein supplements, these were Cladosporium cladosporoides, Spicaria elegans and Mycogone I 103. Cladosporium cladosporoides was found to be toxic when fed to mice at a 50% level in their normal diet. Spicaria elegans and Mycogone I 103 did not reflect any observable toxic effects when fed to mice at a 25% level in their normal diet.

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Section 1

Introduction

Introduction

With the increase in populations in most countries of the world, the problem of obtaining an adequate diet for these populations becomes increasingly acute. In some cases this problem is one of distribution, some countries being in excess of their food requirement, while others are on the threshold of starvation. A survey of the parameters concerned with crop yield and calorific requirements of the populations reflects in general that there is no shortage of food at present or likely to be in the near future. (Gray, W. D. 1962) However, the survey does not reflect that there is a shortage of protein and an excess of carbohydrate in many areas of the world, and this problem may increase in magnitude as populations themselves increase.

Since mans choice of protein is animal protein, as the populations of the world increase so must animal protein production; unless mans eating habits can be changed such that he uses plant protein directly, thus eliminating the plant protein animal protein conversion which is about 20% efficient. A plant such as the soybean which contains 35% protein would probably suffice. However, it is possible to produce more protein per unit area of land with potatoes, a low protein crop, than with soybean a high protein crop. (Gray, W. D. 1962) Thus agricultural economics favour the cultivation of potato crops but man would have to ingest large amounts to meet his daily minimum protein requirements.

Protein production has been increased in many parts of the world by use of intensive farming methods, but many countries are approaching their optimum limits; therefore if arable land is released to graze cattle it may well result in the loss of crops which are essential to our diet. Cultivation of the waste lands of the world for grazing cattle would undoubtedly increase the world supply of protein, however a costly programme in terms of time and money, also mans inability to work as a world wide unit makes it less tangible now or in the near future. Intensive fish farming is possibly another solution to the worlds protein problem, however, the protein of mammals is preferred by many populations to the protein of fish, so at the most it will only serve as a supplement to the main protein diet. Unless the biochemist can accomplish total synthesis of proteins, man must attempt to exploit other organisms directly or indirectly for his source of protein.

In search of an answer to what at first seems an insoluble problem, many biochemists have turned their attention to micro-organisms. Several micro-organisms have as high as a 500 fold growth rate over plants and nearly 1,000 fold over cattle. Given the right environment a single microbial cell, dividing every 20 minutes, could produce 2.2×10^{43} cells in two days. Even though the weight of a single cell is only 10^{-12} grams the total weight of this biomass would be 2.2×10^{25} tons. (Ghose, T. K. 1969). This mathematical interpretation is not obtainable in practice because of limitations of space,

oxygen, substrate supply, temperature and pH. However it does show the potential reproductive power of microbial cells. The production of microbial protein will depend largely on its economic viability, although this will vary from country to country, in general the cost of producing microbial protein must not exceed the cost for producing conventional animal protein if it is to supplement our diets. If the microbial protein is to supplement animal feed, the cost of producing the microbial protein and thus animal protein must be balanced against the economical gain in the release of grazing land for crop production; thus the cheapest form of utilizable substrate for microbial protein must be used. If this can take the form of carbohydrates, which in some countries are in excess of their requirements, by-products or waste from other industries, the production of microbial protein may become a viable economic proposition.

The selection of micro-organisms for the production of microbial protein will undoubtedly depend upon many intergrated factors such as, nutritional requirements of the organism; availability of engineering skills in food technology; and the economic feasibility. (See table 1, Mateles, R. I. and Tannenbaum, S. R. 1968)

Factors Relevant to the Choice of Micro-organisms for the Production of Microbial Protein.

Nutritional

(i) Amino acid pattern

(ii) Protein digestibility

(iii) Effects of extraneous material

(1) Cell wall

(11) Nucleic acid bases

(111) Other

(iv) Protein content

Economic and Technological

(i) Yield of protein per unit of substrate

(ii) Cost of substrate

(iii) Cost of nutrients other than carbon substrate

(1) Oxygen

(11) Minerals

(111) Vitamins and other growth factors

(iv) Productivity-mass of protein per unit volume - day

(v) Cost of sterilization

(vi) Recovery cost - type of recovery operation

Food Technology

(i) Flavour

(ii) Texture

(iii) Solubility

(iv) Colour

(v) Possible processing to improve nutritional organoleptic, or technological qualities

Since it is most unlikely that an organism will be found that will produce an ideal protein containing all the essential amino acids in the correct ratio, the main objective should be to produce an adequate protein at a low price. However in order for a protein to be most useful it must not deviate drastically from the ideal protein for then the biological value of the protein is

lowered. It is possible that two or three micro-organisms may be found which have supplementary amino acid patterns, therefore by mixing them a protein with a high biological value could be obtained. The protein content of micro-organisms is variable, it not only depends upon species variations but may also be influenced by cultural conditions. If micro-organisms are grown in media with high C:N ratios (excess carbohydrate) they will tend to lay down energy reserves in the form of glycogen or poly-β-hydroxybutyric acid in the case of bacteria; fats, lipids and possibly trehalose in the case of fungi, therefore the protein content is reduced. This can be avoided by culturing the organisms under conditions in which the growth is limited by the energy source.

The crude protein content of micro-organisms are usually expressed in terms of Kjeldahl nitrogen (N x 6.25). For many reasons this usually results in overestimates of the true protein content of the micro-organisms. This is because there is a considerable amount of Kjeldahl - reactive nitrogen in materials other than proteins, for example in purines and pyrimidines of nucleic acids, in amino sugars contained in the cell wall of the micro-organisms. Even estimations of protein content by the sum of the individual amino acids are liable to errors since there may be amino acids contained in unnatural peptides or cell wall material, also certain of the amino acids may be present as the D-isomer form which will lead to high values for amino acid content. The cell wall may also be composed of undigestable peptidoglycans, lipopolysaccharides, mucopolysaccharides or chitin, thus protecting the interior of the cell from digestive enzymes, and thus reducing the availability of the proteins, also the cell wall forms the majority of the waste material in microbial protein. (Mateles, R. I. and Tannenbaum, S. R. 1968).

The amino acid composition of a micro-organism is usually a qualitatively and quantitatively stable characteristic of the cell under fixed growth conditions, but the amino acid content varies slightly among different strains and under different environmental conditions (Stokes, J. L. and Gunners, M. 1964). The lysine content of microbial protein in many cases is found to be adequate and in some cases extremely high, (Reusser, F. et al. 1957; Tannenbaum, S. R. and Mateles, R. I. 1968) thus lysine deficient proteins could be supplemented with microbial protein. The pattern and quantity of amino acids and essential amino acids in microbial protein or any protein determines the nutritive value of that protein; this pattern or proportion of essential amino acids has been designated as the amino acid balance.

The concept of amino acid balance was introduced in 1915 by Osborne and Mendel and has been extremely useful for the evaluation of protein quality. Implicit in the concept of amino acid balance was the assumption that although the efficiency of utilization of an unbalanced protein is low, the surplus of amino acids which cannot be used for protein synthesis by an animal, exerts no adverse effect. However, since 1955 (Mauron, J. 1969) evidence has been accumulating that under special conditions, such as in diets with a protein content inadequate to support maximal growth, a surplus of essential amino acids may actually cause some deleterious effects, this has been referred to as amino acid imbalance.

The concept of amino acid imbalance originated from observations made by Elvehjem, C, A. & Krehl, W. A. in 1955. They found that when gelatine or threonine was added to a 9% casein diet lacking niacine, was fed to rats, it resulted in a severe depression of their

growth rate. However this could be prevented by a supplement of niacine or tryptophan. This was considered to be the result of an amino acid imbalance due to the addition of a non-balanced protein like gelatine or an essential amino acid like threonine. Similar results have been observed with other experimental animals on a variety of diets, such as the D-amino acids in the cell walls of microbiol protein which have been shown to cause reductions in growth rates in chicks. (Sugahara, M. & Morimoto, T. 1969). Thus the choice of micro-organisms for the production of protein does not depend on their total protein content, but rather on their amino acid spectra and available protein.

Man has consumed microbiol protein consciously or unconsciously since his existence upon this planet. Bacteria and microfungi and no doubt macrofungi on various foodstuffs; protozoa, algae and microfungi in drinking water. The discovery that certain organisms such as some members of the fungi were palatable and non toxic was probably made early in mans evolution, thus these organisms or products derived from them form part of our present diet. Although man still consciously or unconsciously consumes microbiol protein the amounts are comparatively small and insignificant relative to the proposed programme of feeding microbiol protein to mammals. This itself poses a problem, for if the micro-organisms concerned normally produce toxic metabolites in minute amounts, such that when a micro-organism is ingested in small amounts and produces no ill effects in mammals, the effect of high concentrate feeding may well be detrimental to the mammals concerned, since the toxic metabolites will also be in greater concentration. Also the environments imposed upon the micro-organism for the production of large quantities of protein may be alien to the micro-organism and may

induce the production of toxic metabolites which are not normally produced. Any microbiol protein fed in excessive amounts to homo-sapiens will prove to be toxic due to the increase in the concentration of purines and pyrimidines in the microbiol concentrate. This toxic effect of the purines and pyrimidines is the result of their detoxification which can cause an elevation of the blood uric acid to detrimental levels which may result in precipitation of uric acid crystals in ones joints or the formation of stones in the urinary tract, this effect would be greater in those individuals with a genetic tendancy to excess production of uric acid. The Medical Research Council has found that 15 grams of dried yeast per day can safely be consumed before any harmful effects due to nucleic acids occurs. (Bunker, H. J. 1968). Edozien, J. C. (1970) found that individuals on a dietary intake of 135 grams of yeast per day (containing 8.7 grams of nucleic acid) did not suffer any gastrointestinal disturbance; however they found that a daily intake of approximately 3 grams of nucleic acid resulted in high levels of urinary uric acid because normal individuals on ordinary diets excrete less than 600 mg of uric acid per day; they suggested a dietary intake of 2 grams of nucleic acid would involve little risk of stone formation in the urinary tract and slightly higher levels would probably pose no hazards as long as urine volumes and pH are not abnormally low. It therefore appears that microbial protein must be used to supplement the main diet in homo-sapiens in small quantities unless the nucleic acid content of the microbial protein can be reduced. It is obvious that such a method for the removal of nucleic acid from microbial protein must be inexpensive and not destroy or contaminate the microbial protein

in any way. One such method is that of Maul, S. B. & Tannenbaum, S. R. (1970) who demonstrated on the laboratory scale that the nucleic acid content of the yeast Candida utilis can be reduced from 7% to 1.0 - 1.5% by a three step heating process which involved passing the yeast through a 56 cm long and 1/32" dia. steel tube.

It is highly unlikely that the populations of the world in this decade will find microbiol protein substituted for animal or fish protein, except in the Western hemisphere where it will no doubt exist until the novelty subsides. The almost immediate and useful benefit is no doubt the supplementation of cattle feeds, since they are able to convert uric acid to the more soluble allantoin, and so the nucleic acids need not be removed. This and the other methods discussed for increasing the worlds supply of protein may suffice while other extensive and far reaching investigations are carried out.

The micro-organism which has received most attention from as far back as the days of Pasteur is yeast. Various strains of yeast have been studied in the fermentation industries, mainly only in brewing. But now the role of yeast is being reviewed with new vigour in terms of its growth potential and protein content.

Much of the recent research on yeast growth for protein production has been concentrated on the use of petroleum based substrates. It has been demonstrated that Candida lipolytica and related yeasts can be grown on paraffinic petroleum (Champagnat, A. 1965) and straight chain hydrocarbons including deparaffinized gas oil (Laine, B. M. 1965).

Bacteria have also been exploited for protein production. They have been grown on molasses and inorganic salt medium,

(Stokes, J. L. 1955) also on highly purified C_{13} - C_{19} normal alkenes (McNab, J. G. and Rey, L. R. 1966).

Algae also provide food for thought, but although they can be grown autrophically it is still expensive and presents many technological problems; (Vincent, W. A. 1968) also the proteins are poorly utilized when the algae is fed intact to animals, this is thought to be due to the resistant cell wall of the algae (Hedenskog, G. and Lennant, E. 1969). Although algae do not require a carbohydrate or hydrocarbon substrate, attempts have been made to culture them on such substrates (Humphrey, A. E. 1970).

When growing micro-organisms on hydrocarbons, residual amounts collect between the membrane and cell wall; however the British Petroleum research team has reduced this amount from 2% to 0.1% and has proved it to be non toxic with animals. (Bunker, H. J. 1968). However, Hunkova, Z. and Fencl, Z. (1970) discovered that fatty acids and toxic intermediates arise during the cultivation of yeast on hydrocarbons.

In recent years many workers in the field of microbial protein have turned their attention to the fungi. Interest in fungi as a source of protein probably originates from the investigations of Takata (1929), (Thatcher, F. S. 1954) who showed that the mycelium of Aspergillus oryzae contained 38% protein and was also a rich source of B vitamins. In general the bacteria, yeasts and algae seem more suitable than fungi, which grow more slowly and are considerably lower in protein content. However the great advantage fungi have is that they can be recovered from the fermentation media by relatively simple operations. Also many of the fungi that are being investigated are edible fungi, thus

eliminating preliminary toxological investigations. Many toxic fungi are known, thus they can automatically be eliminated from consideration for protein production, also considerable knowledge of fungal metabolism has been accumulated compared with that of bacteria and algae, and the ease with which fungi can be cultured on various media, especially carbohydrate makes them one of the most suitable organisms to be exploited for protein production.

Studies of the nutritional and growth characteristics and parameters effecting such characteristics of fungi have been investigated by various workers with interests directly or indirectly related to the production of fungal protein. The effect of concentrated sugar solutions on some fungi was investigated by Bezssonof, N. (1919) who showed that such solutions caused the production of fruiting bodies in the case of Penicillium glaucum, Aspergillus oryzae and Rhizopus nigricans; in addition to this it was found that Aspergillus oryzae initiated alcoholic fermentation. In the case of Aspergillus and Penicillium it has been found that fructose is utilized more readily than glucose. (Brannon, J. M. 1923).

Studies of the nutritional requirements of several fungi was carried out by Hochapfel, H. H. (1925) who concluded that the optimum temperature for fungal growth was between 26 - 28°C, optimum pH between 4.3 - 5.5, but the pH of all fungal cultures investigated increased in the prescence of all carbon sources except dextrin, lactose and arabinose, also fungal growth was found to occur in the presence of 60% sucrose or dextrose; the utilization of nitrites occurred only under alkali conditions whereas nitrates and ammonia were readily utilized as a nitrogen

source, however ammonium succinate, glycocoll and bactopeptone were utilized both as carbon and nitrogen sources. Fungi are also able to utilize amino acids as a nitrogen source; Dedic Koch, G. A. 1957 discovered that mixtures of amino acids were more satisfactory in supplying nitrogen for protein formation and growth than individual amino acids. This is in keeping with the work by Thorne, R. S. W. (1944) who studied a similar effect in relation to yeast. Although fungi are able to utilize various compounds as carbon or nitrogen sources, this utilization is usually accompanied by morphological changes; (La Fuze, H. H. 1937) however such morphological changes and physiological changes can also be brought about by the presence or absence of heavy metal ions. (Foster, J. W. 1939).

Attempts have been made over the past few years to promote the growth of fungi by the addition of various compounds to the basal media. McVeigh, I. & Burkholder, P. R. (1940) discovered that the optimum growth of the members of the Phycomycetes could be obtained when ammonium nitrate and fumaric acid was added to the basal media; thiamin (Vitamin B_1) was added to the media in these experiments but it did not appear to be necessary for optimum growth of the Phycomycetes. Although many fungi are able to grow in a vitamin free medium the addition of vitamin B_1 or its components usually enhance fungal growth. (Hawker, L. E. 1944). It has been claimed by Shropshire, R. F. (1949) that the growth of fungi, in fact any micro-organism can be " accelerated by treating the cultures with compressional wave vibrations at a power level insufficient to disrupt the cell structure ". However the evidence for this is rather scanty. In some species of fungi

growth promoting effects have been observed upon the addition of purines or other nucleic acid constituents. (Fries, N. 1954). This is not surprising if the nucleic acids are assimilated intact, for they are indispensable for the growth of any organism that relies upon deoxyribonucleic acid and ribonucleic acid for replication. Plant hormones have also been used to promote the growth of fungi, and in some instances the reverse has occurred. Leelerathy, K. M. (1968) discovered that indole acetic acid and 2 - 4 dichlorophenoxy acetic acid at 400 ppm. reduced fungal growth, whereas gibberellic acid at 1 - 5 ppm. caused some stimulation of fungal growth. Not only have plant hormones, amino acids, nitrates and nitrites etc. been used to promote fungal growth but bizarre chemicals like 2 - oxo- 4 methyl - 6 - ureidohexahydropyrimidine or its hydrolyzate 2 - oxo - 4 methyl - 6 - hydroxyhexahydropyrimidine have been used with some success (Carp, C. 1968).

In comparison with the volumonus literature of the nutritional and growth characteristics of fungi that of metabolism is rather scanty; although some compounds found in the fungal mycelium may be attributed to certain physiological or biochemical states others can not. The majority of the work has been centered around nitrogen utilization, sulphate metabolism, secondary metabolite and toxin production, but even so, many questions are left unanswered.

Kostychev, S. and Tsvetkova, E. (1920) investigated the utilization of nitrates and nitrites by Aspergillus niger and Mucor racemosus and found that both these organisms reduce nitrates and nitrites to ammonia (NH_3^+) from which they synthesise amino compounds. They also observed that the reduction of the nitrate to nitrite can be brought about without the addition of

sugar, but further utilization of the nitrite only takes place in the presence of sugar, also they suggested that the nitrate nitrogen is converted to ammonia (NH_3^+) and amino nitrogen outside the hyphae. This is in direct contrast to the observations of Nonov, N. N. and Tzvetkov, E. S. (1936) . They demonstrated that when a fungus is grown on nitrates the nitrate ion penetrates into the cell and is reduced to ammonia (NH_3^+) and afterwards is disengaged, the reaction one is left to assume would probably be in the order of $3HO^- + 3H^+ + 2NO_3^- = 2NH_3^+ + 2NO_3^-$. Investigations on the pH effect on nitrate absorbtion by Aspergillus niger were carried out by Itzerott, D. (1936) who discovered that nitrates are only utilized at a pH below 3.0 and then only when insufficient ammonia (NH_3^+) was present; however this behaviour could not be correlated with the optimum pH for nitrate reduction. It is not surprising that in the presence of sufficient ammonia (NH_3^+) nitrate utilization is reduced, for there is one less step in the metabolic pathway. It has been suggested that in the absence of organic and inorganic nitrogen fungi are able to utilize atmospheric nitrogen; however if there is such a nitrogen deficiency in the media, protein, ribonucleic acid, deoxyribonucleic acid, riboflavine, pyridoxine, cobalamin and biotin contents are found to be reduced. (Kleber, H. P. et al. 1968) If fungi were able to fix atmospheric nitrogen a reduction in the mentioned cell constituents would not occur, unless the fixation of atmospheric nitrogen was at such a low level such that it was just sufficient to maintain cellular unity. However experiments with isotopic nitrogen N^{15} have not verified that fungi are able to fix atmospheric (free) nitrogen. (Tabak, H. and Cooke, W. M. B. 1968).

Sulphate metabolism in fungi is rather complex for as a sole source of sulphur they can utilize sulphate, sulphite, thiosulphate, sulphide, choline sulphate, cystine, methionine and many other sulphur compounds; it is obvious that they therefore must possess enzymes systems capable of interconversion of a large number of sulphur compounds; thus if a given reaction is shown to occur it can not be concluded that this reaction is a part of the normal sulphate utilization mechanism. According to Johnson, M. J. (1964) the primary reaction undergone by the sulphate ion in fungal metabolism, or any other micro-organism, is its conversion to adenosine 5' - phosphosulphate (APS) by the following reaction:

$$ATP + SO_4^{2-} \longrightarrow APS + PP$$

This reaction is strongly endergonic and is caused to proceed in the forward direction only because both products are removed as quickly as they are formed; the pyrophosphate is removed by a pyrophosphate phosphohydrolase, and the adenosine 5' - phosphosulphate (APS) is phosphorylated by adenosine 5' triphosphate (ATP) to produce 3' - phosphoadenosine - 5' - phosphosulphate (PA PS), from which the sulphate group can be transferred by adenosine - 5' - triphosphate (ATP) and sulphate adenylytransferase to a wide variety of compounds.

Most if not all fungi are able to synthesise additional chemicals apart from those which are necessary for the existance of the fungus, these are the secondary metabolites. Secondary metabolite production in fungi is generally a species characteristic, but there is a great diversity in the molecular constitution of secondary metabolites. Little is known of their metabolic function, and in many cases the formation of secondary metabolites is associated with the availability of a high level of carbohydrate in the culture medium (Bentley, R. and Campbell, I. M. 1968).

Apart from the secondary metabolites some fungi are capable of producing other metabolites known as toxins. These toxins, which in some cases may be secondary metabolites are known as mycotoxins.

" There is a substantial amount of information on toxins produced by fungi, but in the majority of cases the causative compounds have not been isolated nor their structures established ". (Borker, E. et al. 1966). However an important group of these toxins are those which belong to the green and white species of Amanita which are responsible for the wellknown mushroom poisonings. The toxic substances in this case are peptides which can be divided into two groups, the phallotoxins and the amatoxins. The structures of these toxins have been elucidated by Wieland, T. (1968). These toxins contain several unusual amino acids and combinations of the common protein amino acids.

Very little interest had been directed to mycotoxicoses in man until the discovery of a carcinogen, aflatoxin produced by Aspergillus flavus. ~~Four~~ Thirteen compounds are known, however the most commonly occuring ones are aflatoxin B_1, B_2, G_1 and G_2; B_1 and B_2 show blue flourescence and G_1 and G_2 green flourescence under ultra violet light, all of the compounds have a furano coumarin structure. The production of aflatoxins by Aspergillus flavus can be stimulated by amino acids such as glutamic acid, proline, leucine, threonine, tyrosine and tryptophan, especially under aerobic conditions for the production of aflatoxin is aerobic.

General furano coumarin structure of aflatoxins for B_1, B_2, G_1 and G_2

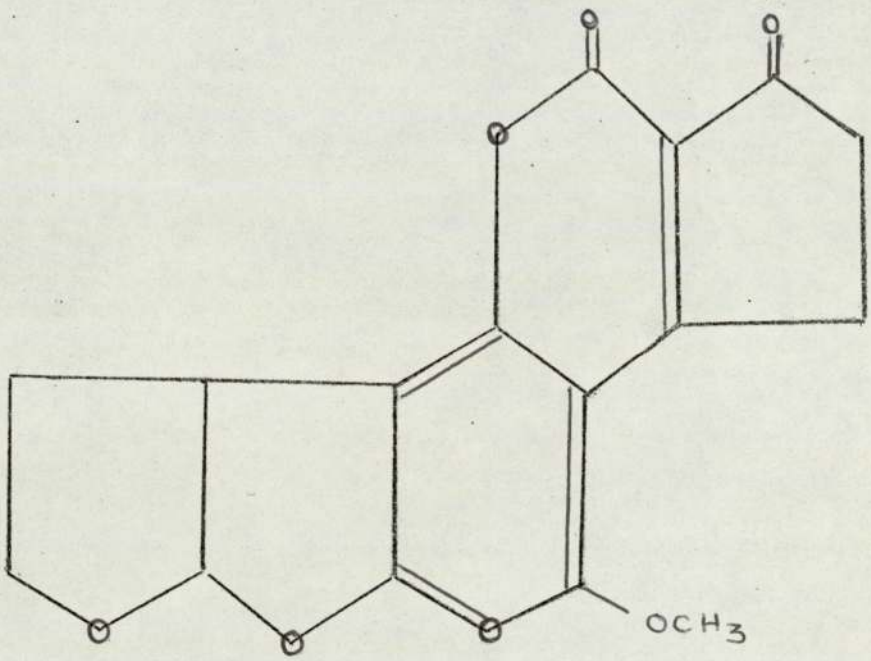


Fig. 1.

Since fungi are aerobic organisms; the qualitative relationship between growth and oxygen demand varies considerably between species of fungi, however this does not mean that fungi will not metabolise carbohydrates anaerobically (fermentation) for this can be accomplished by preformed cell material, but growth at the expense of fermentative metabolism does not occur. The growth stimulating range and deleterious effects produced by the lack of oxygen or excess concentrations of carbon dioxide vary with different species and strains of fungi; however carbon dioxide is indispensable for fungal metabolism for the production of di and tri carboxylic acids, organic acids and vitamins (Tabak, H. H. and Cooke, W. M. B. 1964). It was mainly the production of these organic acids and vitamins by the fungi that first stimulated the fermentation industry in their commercial exploitation of the fungi.

Up until the beginning of World War II commercial production of fungi which was mainly for fat production was carried out in shallow-pans, (Lindner's process) even laboratory investigations were mainly conducted using surface cultures. During the later part of the war a successful submerged vat process was developed by Damm (1944) (Robinson, R. F. 1952) which gave results superior to Lindner's process. Because of this and the following success of submerged fermentation in the antibiotic field the idea of growing higher fungi for their mycelium developed. Research was started by several investagators, and they found some strains of mushrooms (Basidiomycetes) were adaptable to this method of cultivation (Robinson, R. F. and Davidson, R. S. 1959). Since then many investigations have been carried out on submerged cultivation of fungal mycelium using various types of media.

Humfield, H. (1948) has shown Agaricus campestris (mushroom) mycelium can be grown in asparagus butt juice, and press juice from pear waste; however the addition of inorganic salts was essential in the pear juice medium. He also found that fairly good yields could be obtained in media consisting of monosodium glutamate, dextrose, and inorganic salts. Also the mycelium of Agaricus blazei has been grown in fruit juice such as citrus press water, orange juice and even synthetic media. (Block, S. S. et al. 1953).

Substrates such as waste sulphite liquor and molasses have been used to grow Tricholoma nudum; (Reusser, F. et al. 1958)[a] it was found that the organism would utilize some of the non-reducing sugars at an optimum pH 3.5 - 4.5, the nitrogen supplements were ammonium tartrate, a nitrate and urea; however it was found that the nitrate and urea depressed the protein content and altered the fungus morphology. In the same year Reusser, F. et al. (1958)[b]

showed that ten strains of Basidiomycetes, including four strains of Agaricus campestris could be grown in synthetic medium; it was discovered that high nitrogen concentrations in the media favoured protein production, whereas reduced nitrogen concentrations induced fat formation. This effect of nitrogen concentration on fungal protein was also observed by Falanghe, H. (1962) when he investigated the growth of ten mushroom cultures grown in vinasse, monopotassium phosphate, magnesium sulphate and ammonium sulphate; when the concentration of ammonium sulphate was varied so did the protein content of the fungal mycelium, but this effect was not observed when the concentration of the inorganic salts in the media was changed. Falanghe, H. et al. (1964) also investigated the growth of eight species of mushrooms in dehulled undenatured flakes of soybean whey in malt extract broth. It was found that soybean whey concentrate increased growth but reduced the protein content of the mycelium; of the seven species of fungi inoculated in soybean whey media containing different ammonium salts only six developed mycelia; addition of ammonium acetate to the media completely inhibited the growth of some of the fungi. It was found that Tricholoma nudum exhibited slurry or yeast like growth in whey media containing ammonium sulphate and ammonium tartrate, whereas with ammonium acetate, formation of spheres 5 - 8mm in diameter was observed.

An intensive survey into the growth and nutrition of the wood rotting Basidiomycetes was carried out by Jennison, M. et al. (1955) these consisted of brown and white rots; observations were made of growth and nutrition in malt extract and synthetic media. Maximum growth of the 42 species involved was obtained in about

two weeks, pH values as low as 1.5 were recorded in the growth media. Two of the species had no vitamin requirements, but most of the others were deficient for thiamine. None of the organisms required organic nitrogen, but however, growth was usually greater with organic nitrogen than with ammonium salts. No differences between the brown or white rots was observed in terms of nutritional characteristics.

Other workers in the field of fungal protein have been more speculative in their choice of fungi and media. Gray, W. D. et al (1963) studied the growth of some members of the Fungi Imperfecti in sea water medium. The Fungi Imperfecti have a great advantage over the Basidiomycetes since their growth rate is higher. It was observed that all of the selected Fungi Imperfecti were capable of growing in sea water, and in some cases it enhanced their growth. Gray, W. D. et al. (1963) concluded that the beneficial effect of the sea water was probably due to the relative concentrations of magnesium and calcium ions in the sea water. Gray, W. D. et al (1964) and his co-workers furthered their work on the Fungi Imperfecti as a potential protein source by screening 175 isolates and determining their economic coefficients; (EC = unit weights of carbohydrate required to produce one unit weight of dried fungus tissue) crude glucose was supplied as the carbon source and ammonium nitrate as the nitrogen source. Following this work Gray, W. D. and Abou-el-Seoud, M. O. (1966)[a] investigated the possibility of using a media containing sweet potato as the carbon source for the growth of some members of the fungi Imperfecti. The crude protein estimation (Kjeldahl nitrogen x 6.25) of the selected fungi Imperfecti varied between 21 and 37 per cent, the

maximum yield was 81.2 pounds of dried mycelium per 100 pounds of sweet potato; thus the results they obtained illustrate that crude carbohydrate containing materials can serve as raw materials for the fungal synthesis of protein. On the basis of this work investigations were carried out by Gray, W. D. & Abou-el-Seoud, M. O. (1966)[b] on the maximum fungal protein that could be produced from crude carbohydrate materials. They found that growth of fungi in cassava root extract gave the highest economic coefficient.

The calorific values of fungal mycelia has also been investigated by Gray, W. D. and Staff, I. A. (1967). The fungi used were 100 of those used by Gray, W. D. et al. (1964). The results were varied, calorific values were recorded between 3.658 K cal/gm of mycelium to 5.662 K cal/gm of mycelium. However it was concluded that there possibly exists a considerable number of fungi with thermal efficiencies much greater than those recorded.

In comparison with the work carried out on liquid cultivation of fungi, surface and submerged, that of solid state fermentation is rather limited. In solid fermentation the substrate is used in paste form, a fungus inoculum is added and the moist paste is fermented as spaghetti like extrusions to form a vegetable cheese in which the strands are bound together and permeated by threads of fungal mycelium (Brook, E. J. et al. 1969). Stanton, W. R. and Wallbridge, A. (1969) investigated the possible use of starch tubers of cassava as the solid substrate to grow Rhizopus, and found a significant increase in the final protein content of the fungal mycelium.

As mentioned previously (page 18.) submerged cultivation of fungal mycelia was developed during the later part of World

War II, when the shallow pan (Linder's process) was replaced by the submerged process developed by Damm (1944) (Robinson, R. F. 1952). Since then the process has become modified and refined.

The conventional type of fermentation vessels for the submerged cultivation of fungal mycelia are stirred tanks of various capacities. The media that the fungus is grown in is stirred by an impeller or impellers set in the tank beneath the surface of the media. Air is supplied to the media beneath the rotating impeller or at the same level, thus the action of the impeller distributes the air into the media as fine bubbles. The power required to stir the media depends on the size of the impeller, capacity of the tank and the viscosity of the media, which increases as the fermentation proceeds due to the growth of the mycelium. The cost of producing fungal mycelia by this method can prove to be expensive, due mainly to the power requirements, also after each fermentation the vessel must be cleaned and sterilized which involves time and loss of manpower while the vessel is out of production. This problem may be solved to some extent by semi continuous fermentation.

Semi continuous fermentation implies a number of vessels, usually seven in all. The first vessel is inoculated with fungal mycelium and when the fungal mycelium reaches its half logarithmic phase of growth, that is the time taken to produce half its total biomass as determined by the fermentation vessel and nutritive value of the media, it is divided equally between two other vessels of the same capacity and made up to the original volume with fresh media. When the mycelia in these two vessels reach their half logarithmic phase of growth they to are divided

but this time between four vessels and again made up to their original volume with fresh media. These are the final fermentaion vessels and the fermentation is allowed to go to completion. (Fig. 2.).

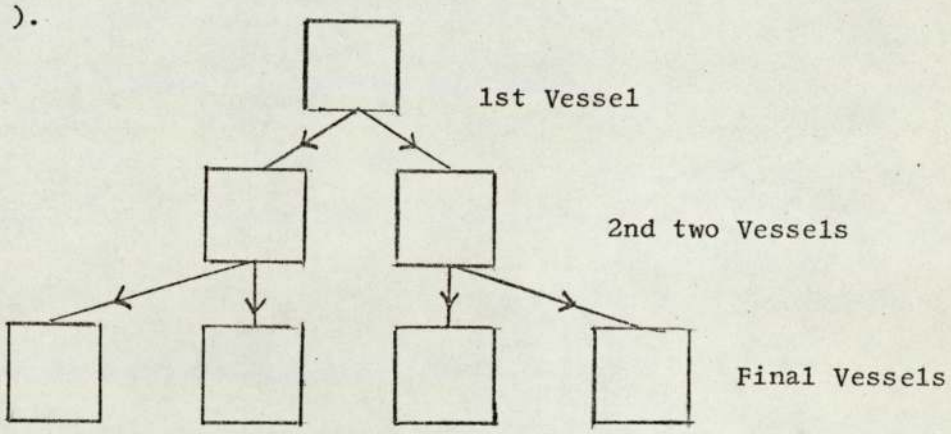


Fig. 2

Therefore as the first vessel is emptied into the second two vessels the first vessel can be cleaned, sterilized and re-started. This process is repeated with the other vessels, thus the vessels are out of production for the minimum amount of time, and man-power is not wasted unless the sequence is broken. Graphically the sequence may be represented as follows (Figs. 3 & 4.):-

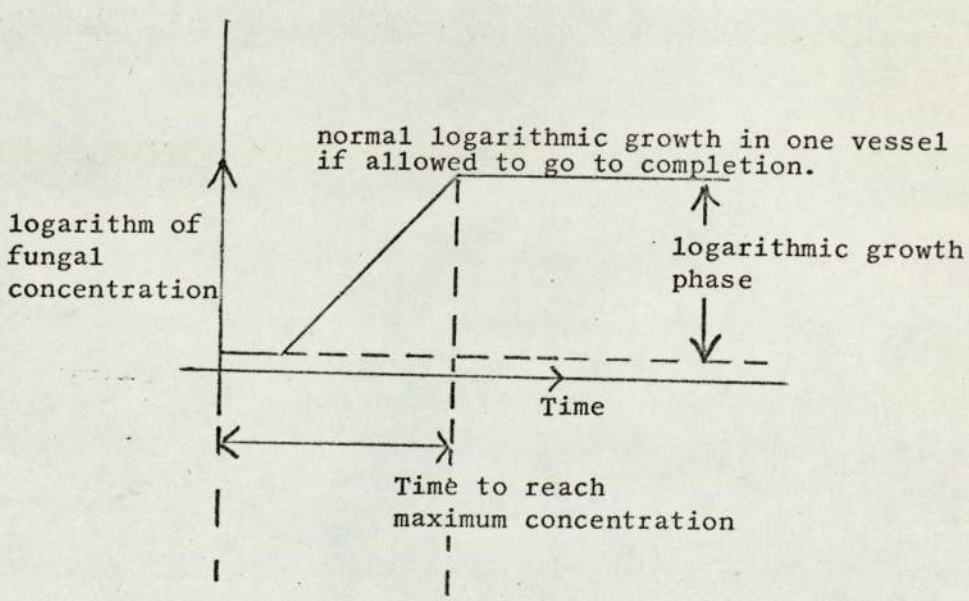


Fig. 3

Graphical effect of dividing the fungal mycelium in its half logarithmic phase of growth.

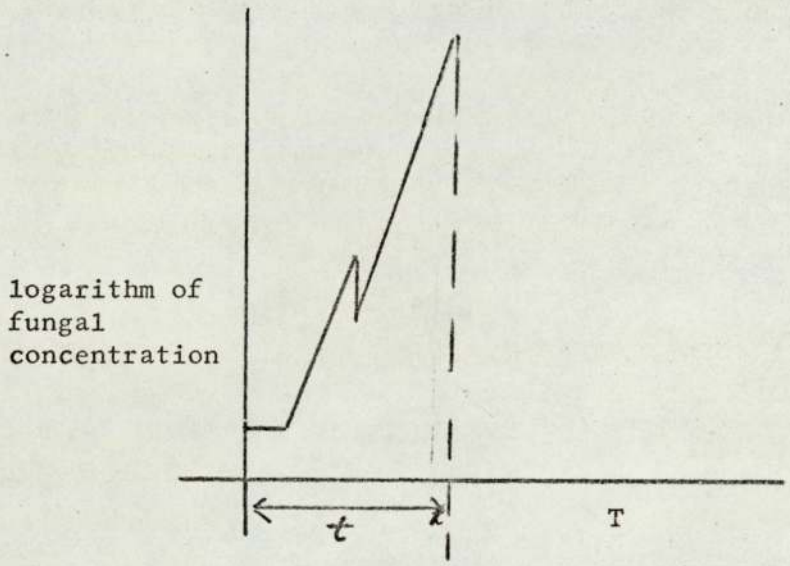
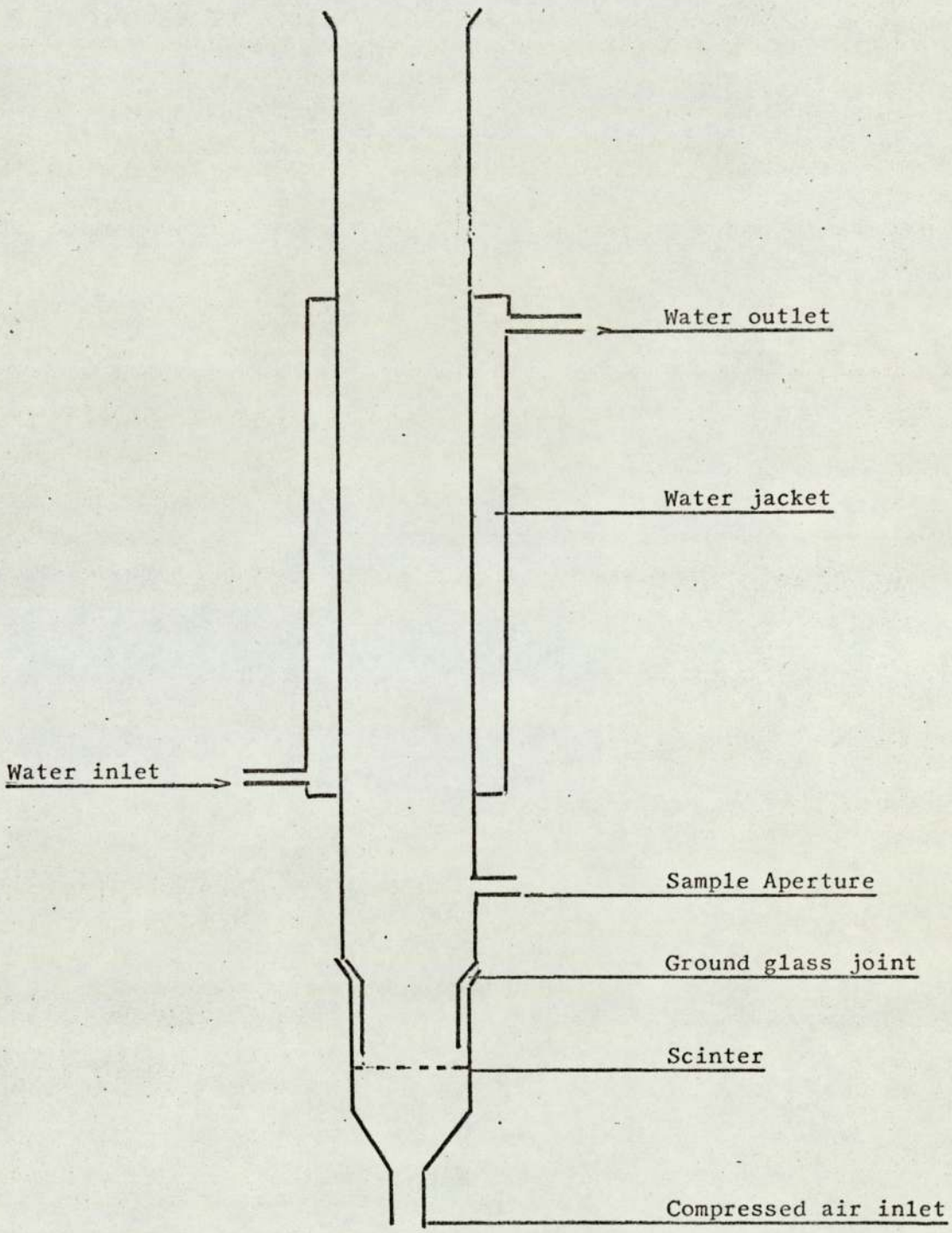


Fig. 4

The time to reach maximum growth in both cases is the same, but in this case double the amount of mycelium is obtained. (Malek, I. and Fencl, Z. 1966). Although semi continuous fermentation is used quite extensively, especially in the brewing industry, the possibility of continuous fermentation has attracted attention.

Continuous fermentation basically consists of supplying media to the fermentation vessel at one point at the same rate that it is being removed at another. The continuous fermentation technique was first conceived by Delbruck, M. (1892) and Barbet, E. (1899), and practical attempts were first made by Rijn, L. A. Van. (1906) and Schalk, H. A. (1906). At that time the available technological skills were insufficient to cope with this new technique, and so it appears that little progress was made. The shape of the continuous fermentation vessel has been of various designs, ranging from round or square stirred tanks in open or closed systems. The fermentation vessel which is of interest at present is the tower fermenter. (Fig. 5.).(page 25,) Greenshields, R. N. & Smith, E. L. (1969); Greenshields, R. N. et al. (1971).



Basic Tower Fermenter

Fig. 5.

This consists of a tall, vertical, cylindrical column which contains the media and the fungi. The media and air are supplied at the base of the tower and the products of the fermentation can be withdrawn from the top. The method is probably more applicable to the recovery of extracellular metabolites than to the recovery of fungal mycelium, however if the fungal mycelium is used for protein and a by-product of metabolism of a useful nature is excreted into the media, this system is ideal. Even if this system is used semi continuouslyit has many advantages; i) no mechanical agitation since this is accomplished by movement of the air through the media via the fine scinter which distributes it as fine bubbles, therefore no large power requirements. ii) easy to clean and sterilize, and maintain sterile. iii) larger cubic capacity per area, thus less land used, therefore the size of a fermentation plant can be reduced in terms of area.

With the tower system, as with any other system, the problem of foam production presents itself. To prevent foaming it would seem obvious that an increase in surface tension would bring about the collapse of the bubbles. However after foam elimination (which can be brought about by only a slight change in surface tension) surface tension may be lower or higher than the initial surface tension (Andrew, K. P. et al. 1968). Agents which eliminate foams must do so with the minimum consumption of chemicals. This requires the formation of an emulsion of the antifoam agent with the media. Some of the antifoam agents which achieve this are mixtures of oil or oleic acid with sulphuric acid, oil with ammonia water or the so called nonsulphurated compounds, which are by-products of synthetic fatty acid production (Andrew, K. P. et al. 1968). In this laboratory silicon is used which brings

about an increase in surface tension.

If attempts are made to grow micro-organisms in liquid or any other type of media in any fermentation vessel, the parameters pH, temperature and aeration are of no minor importance. Temperature control is not a major problem since various heating and cooling elements are available, but these parameters influence each other as well as the resultant growth and metabolic state of the micro-organism. This was demonstrated by Rose, A. H. (1968) who discovered that in the case of yeast a drop in the fermentation temperature caused an increase in ribonucleic acid and protein content of the yeast cell, an increase in cell volume and an increase in the size of the amino acid pool. At a fixed oxygen tension a greater number of unsaturated fatty acids were synthesised, but at a fixed temperature a decrease in the dissolved oxygen tension led to a decrease in the number of double bonds and thus a preferential synthesis for short chain fatty acids. Keto acids particularly pyruvate were excreted by the cells but amino acids were not, in fact the amino acid pool increased when these short chain fatty acids were synthesised.

The one parameter which is affected by most, if not all other parameters is aeration. Fungi are basically aerobic organisms although under certain environmental conditions can survive anaerobically for short periods, but for normal growth oxygen is required. Once the oxygen is in solution it must pass to the cell by the process of diffusion. If the oxygen is not in solution but in the form of bubbles it is conceivable that direct gas to cell transfer might occur. This effect is probably insignificant since agitation intensity has no influence on the mass transfer rate once

it is sufficient to ensure gas bubbles and fungal mycelium are freely suspended (Blakeborough, N. 1967). Fungi, in fact micro-organisms in general, utilize oxygen in solution at a rate which is idependant of the concentration, down to a level of approximately 0.01 - 0.02 m moles/L. Therefore, this value determines the minimum oxygen supply rate which must be maintained. Factors such as viscosity of the media also interfere with oxygen transfer, (Solomons, G. L. 1961) as well as temperature and pH, also the addition of an antifoam agent will affect the uptake of oxygen into solution, therefore indirectly effecting the oxygen transfer rate from the media to the fungal mycelium if the oxygen tension is reduced below 0.01 - 0.02 m moles/L.

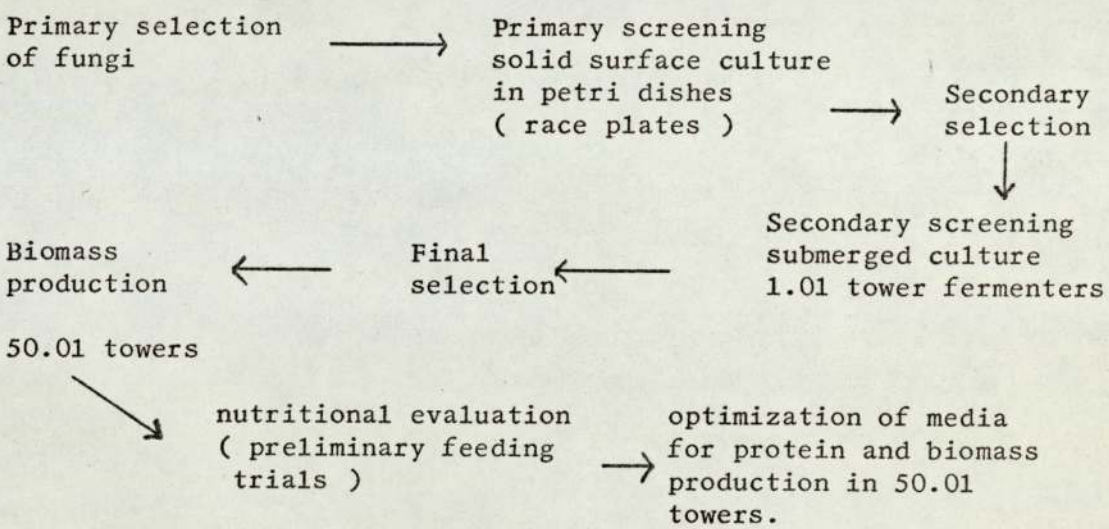
The production of microbial protein may initially create as many problems as it will solve, and no doubt be exploited commercially by Western Technology, but it is well to remember that it is a step towards increasing the worlds protein supply.

Section 2

Theoretical Considerations

Theoretical Considerations.

The object of this investigation was to screen selected fungi from the Basidiomycetes and Fungi Imperfecti for growth, nutritional characteristics, and nutritional value when grown on an aqueous extract of the carob pods of Ceratonia siliqua (from which the seeds had been removed) as the only source of carbohydrates supplemented with (i) Glutamic acid, (ii) Ammonium sulphate, (iii) Glutamic acid and Ammonium sulphate. The screening was carried out on solid surface culture in petri dishes (race plates) and submerged culture using a tower fermenter system (see fig. 5. page 25.). The scheme of screening and selection is presented in the flow sheet below.



The mathematical interpretation of growth rates of micro-organisms grown on a solid substrate (race plates) (primary screening) can result in eroneous statements and conclusions. Thus the rate of growth of the micro-organisms can only be used as an indication of the growth rate of that micro-organism under those conditions prevailing on that particular race plate at that

particular time. No matter how much attention is directed to the preparation of race plates, not all will be of uniform thickness due to gelation of the agar at points of rapid cooling. It is also conceivable that individual race plates will vary from each other in their overall thickness and solidity. The amount of condensation in the closed environment of each race plate will vary due to different races of cooling of the media before gelation; also the place it occupies within the incubator will also affect its overall humidity. Undoubtedly all these factors will affect the characteristics of any micro-organism which in part may be reflected in its growth rate or its special arrangement on the substrate.

It is quite feasable for any micro-organism grown on a minimal medium, particularly fungi to respond to these conditions with a diffuse sparse rapid rate of growth, thus giving the impression that energy is being conserved until sufficient nutrients are found to support a larger biomass. A mathematical interpretation would certainly reflect the micro-organisms rapid rate of growth, irrespective of its biomass. Thus when measuring the rate of growth of a micro-organism, especially fungi on a solid substrate, the type of growth must be taken into account.

Since there are so many variable factors that affect the growth characteristics of micro-organisms grown on solid surface culture (race plates) a statistical approach to the rate of growth of the fungi would be desirable. However in the case of the author where a number of fungi were selected for comparative work a complete statistical approach was not practical in terms of time and physical effort involved. It was therefore decided to conduct the primary screening in triplicate and construct a graph of the average growth against time for each fungus, but at

the same time considering the type of mycelial growth involved when selecting fungi for secondary screening. To obtain a graph of growth against time the area of a circle was considered.

Area of a circle is: πr^2 = Area

Since π is a constant

$$r^2 \propto A$$

(r^2 is directly proportional to the area)

By transplanting circular pieces of a fungal colony of known dimensions into a corresponding aperture on a race plate circular growth of the fungal colony was assured, thus the radius could be obtained.

Although complete statistical analysis was not carried out on the rate of growth of the fungi, it was however carried out on those fungi selected for secondary screening in the tower fermenter, in terms of growth attained on the three different supplemented carob media. The statistical analysis was the ' t ' test, to see if the difference in growth, if any, was due to chance events, or media supplementation since all other parameters were assumed to be equal.

' t ' test.

To test the significance between two means of unpaired series of observations.

$$' t ' = \frac{\bar{x}_1 - \bar{x}_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \qquad n_1 = n_2$$

$$' t ' = \frac{\bar{x}_1 - \bar{x}_2}{S\sqrt{\frac{2}{n}}} \qquad n_1 = n_2$$

in both cases $S = \sqrt{\frac{(x_1 - \bar{x}_1)^2 + (x_2 - \bar{x}_2)^2}{n_1 + n_2 - 2}}$

x = single observations

$\bar{x}$ = mean of observations

n = number of observations

s = standard deviation

Degrees of freedom = $n_1 + n_2 - 2$

Since the primary screening was carried out on race plates, solid substrate surface culture, it would seem that little correllation can be made between this and the secondary screening in submerged culture. This is not strictly true for Trinci, A. P. J. and Gull, K. (1969) have shown that in the case of Absidia giauca the colony growth rate was directly related to its specific growth rate in submerged culture at the same temperature, pH and media concentration. However other factors were involved in the decision to use race plates: (i) speed of screening by race plates was far superior to submerged culture in tower fermenters or shake flasks (ii) greater number of fungi could be screened at one particular time (iii) size of inoculum was constant, (8mm transplants).

The selection of fungi from the primary screening on race plates for secondary screening in submerged culture is not as difficult as it first appears. When aerobic micro-organisms which normally inhabit the surface of solid or semi-solid substrates are transferred to submerged culture, various results are possible in terms of growth rates (i) rate of growth decreased (ii) rate of growth increased (iii) lag phase increased (iv) lag phase reduced (v) combinations of all four. Since submerged culture provides a hostile environment mainly in terms of low oxygen tension, it would seem more likely that the rate of growth of the fungi would be reduced and its lag phase increased; but this is not always the case and the reverse can occur. However if the extremely rapid growing fungi are selected from solid substrate

surface culture for submerged culture and the extremely slow growing ones rejected, since if all things being equal it is most unlikely that an extremely slow growing fungus will have its growth rate increased and its lag phase reduced to such a degree where it can compete with a rapid growing fungus with a reduced growth rate and an increased lag phase.

Since it is known that a high concentration of carbohydrates will enhance lipid and secondary metabolite production in fungi, (see pages 5 & 15.) a medium containing a low concentration of carbohydrates was used. In effect this was limiting the energy source, thus excess carbohydrate was not available for lipid and secondary metabolite production. The optimum concentration of carbohydrates for fungal growth will no doubt vary with species of fungi; however 4 - 5 gram per cent concentration of total carbohydrates was chosen as the concentration of carbohydrates to be used in this investigation. This concentration of carbohydrates has been found to be most suitable for many of the experiments conducted on production of microbial protein in this laboratory. It is not only the carbohydrate concentration that affects lipid and secondary metabolic production, but also the type and amount of nitrogen present in the media. This carbohydrate - nitrogen relationship is expressed as the C:N ratio, and low C:N ratios favour protein synthesis. (see page 5.). Since the fungi will break down glucose polymers to individual glucose units to be utilized in glycolysis, also fructose can be converted to glucose and thus enter glycolysis, the amount of carbon present in the media is therefore calculated as glucose carbon based on the g% of total carbohydrates present in the media.

In this investigation two nitrogen sources were used, ammonium sulphate and glutamic acid. The reason ammonium sulphate was

selected is as follows. Firstly ammonia has been found to be a good sorce of nitrogen (see page 14) in supplying the amine (NH_2) group for amino acid synthesis, hence protein synthesis. The ammonium sulphate will exist as ions in solution,

$$(NH_4)_2 SO_4 \longrightarrow 2 NH_4^+ + SO_4^{2-}$$

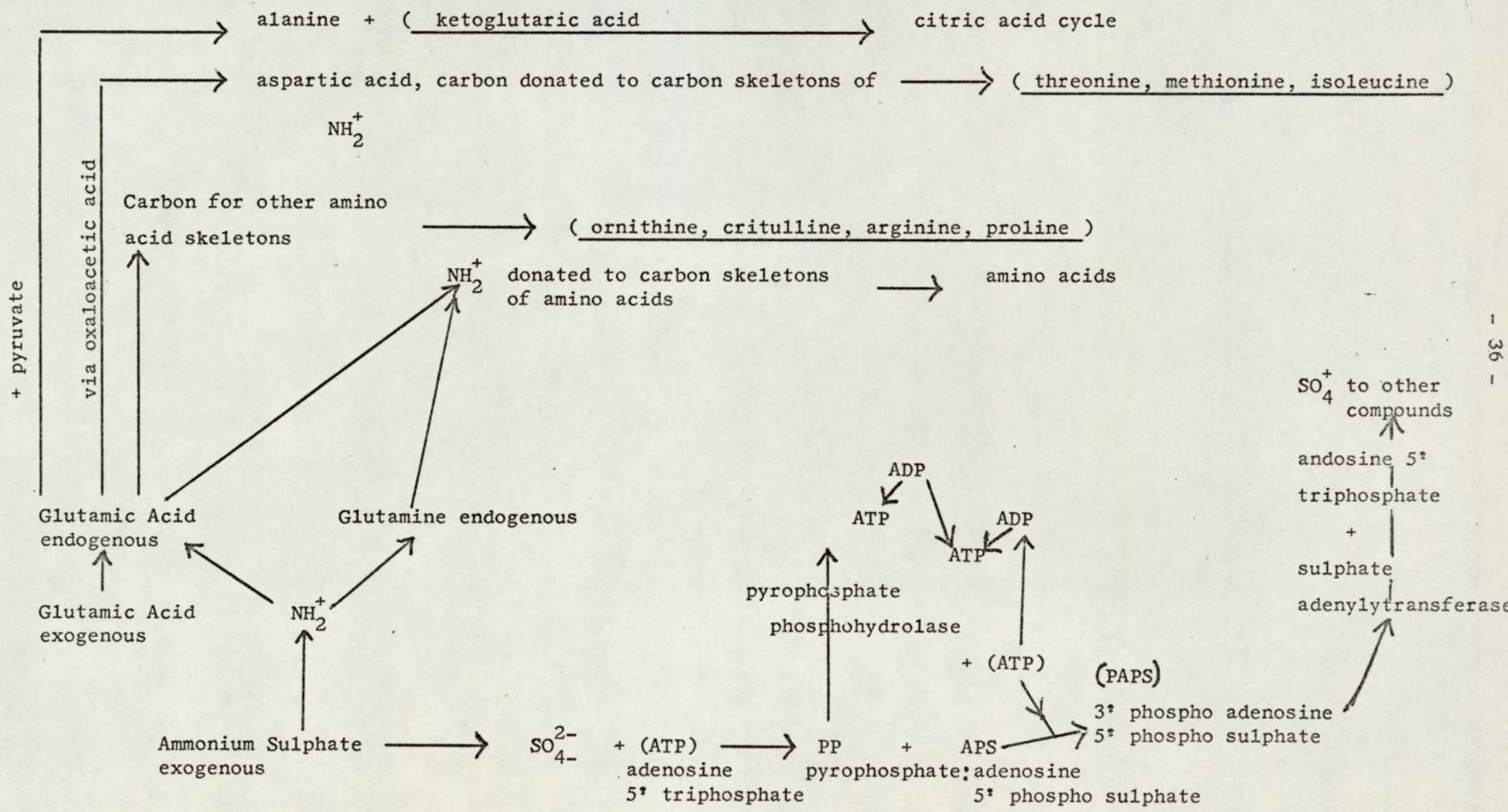
Thus the ammonium ion will supply the amine (NH_2) group for amino acid synthesis, the sulphate ion (SO_4)$^{2-}$ will enter into the sulphate metabolism of the fungi. (See page 15.). This may therefore be a supply of sulphur for the sulphur containing amino acids. The reason glutamic acid was selected as a nitrogen source was based on previous investigations carried out by the author on the effect of glutamic acid on the growth rate and protein content of yeast. Since yeast is a fungus there must be some basic metabolic similarities with other fungi.

In yeast glutamic acid and glutamine are the only amino acids to derive their amino nitrogen directly from ammonia (Sims, A. P. and Folkes, B. F. 1964). Sims, A. P. and Folkes, B. F. (1964) also demonstrated that the carbon skeleton of glutamic acid could be contributed to the formation of ornithine, critulline, arginine and proline in yeast. Now since glutamic acid may also be converted to aspartic acid via oxoloacetic acid, then the carbon skeleton of aspartic acid may contribute to the formation of threonine, methionine and isoleucine. Glutamic acid can combine with pyruvate to form alanine and ketoglataric acid, which is an intermediate of the citric acid cycle. Thus the possible utilization of pyruvate in the case of yeast will in part prevent the production of alcohol via acetaldehyde, at the same time in yeast and fungi the ketoglutaric acid may supplement the citric acid cycle. It has

been demonstrated by Jones, M. et al (1969) that transaminase systems operate within the yeast cell, showing that the theory of intact assimilation of amino acids into yeast protein does not occur, although intact assimilation of the amino acids do, which undergo transaminase reactions so that their α amino nitrogen is transferred to other amino acid skeletons, also all the amino acids in yeast protein can derive their α amino nitrogen from glutamic acid or glutamine mainly by transamination reactions. If this holds true for other fungi then the protein content of the fungi should be enhanced and contain a larger proportion of protein with those amino acids derived from the carbon skeleton of glutamic acid and aspartic acid.

The effect of glutamic acid on the growth of yeast was to increase the duration the yeast spent in its logarithmic growth phase and to increase its protein content. It is plausible that this extra duration in its logarithmic growth phase was the period in which the yeast produced this extra protein. It would appear that the rate of growth is reduced to enable this protein to be synthesised.

The following metabolic chart depicts the possible metabolic fate of the exogenous ammonium sulphate and glutamic acid in fungal metabolism.


alanine + (ketoglutaric acid
citric acid cycle
aspartic acid, carbon donated to carbon skeletons of
(threonine, methionine, isoleucine)
NH_2^+
Carbon for other amino acid skeletons
(ornithine, critulline, arginine, proline)
NH_2^+ donated to carbon skeletons of amino acids
amino acids
+ pyruvate
via oxaloacetic acid
Glutamic Acid endogenous
Glutamine endogenous
Glutamic Acid exogenous
NH_2^+
Ammonium Sulphate exogenous
SO_{4-}^{2-}
+ (ATP)
adenosine 5' triphosphate
PP + pyrophosphate
APS adenosine 5' phospho sulphate
ADP
ATP
ADP
ATP
pyrophosphate phosphohydrolase
+ (ATP)
(PAPS)
3' phospho adenosine 5' phospho sulphate
SO_4^+ to other compounds
andosine 5' triphosphate
+
sulphate adenylytransferase

All the fungi screened and selected were grown at 30°C since this is approximately the average atmospheric temperature in Cyprus where successful fungi are to be grown for biomass production. Fungi requiring a lower temperature for growth would necessitate the installation of extensive cooling aparatus in a production plant, thus elevating the cost of fungal biomass production.

Section 3

Chemical Analyses

Chemical Analyses

The following chemical analyses are those which have been used extensively, or at least more than once in this investigation, and have therefore been grouped in one section to avoid repetition.

3.1 Nitrogen determinations and protein estimations

The nitrogen determinations were carried out by use of the standard Kjeldahl method, the catalyst used was a mixture of sodium selenite, potassium sulphate and copper sulphate in a ratio of 1 : 116 : 40 respectively. Protein estimations were obtained by multiplying the total nitrogen by 6.25. (On average proteins contain 16% nitrogen) Markham, (1942); Greenshields, R. N. (1965).

3.2 Carbohydrate estimations

In the estimation of carbohydrates the Fehling's and the Anthrone methods were used. The Fehlings method was that of Lane, J. H. and Eynon, L. (1923) except that the hydrolysis of the samples for total carbohydrate estimations were carried out at 90 - 95^{o}C for 10 minutes and for every 10 ml of sample 1 ml of concentrated hydrochloric acid was added; the sample was then cooled and made alkali with 40% sodium hydroxide and made up to a final known volume. The resulting titrations were read off against a calibration curve. The calibration curve was constructed using samples of glucose of known concentrations. To estimate the reducing sugars in a particular sample the hydrolysis procedure was omitted.

The standard Anthrone method was that of Seifter, S. et al. (1950), for the estimation of total carbohydrates. The calibration curve was based on glucose. The Anthrone method has greater sensitivity than the Fehlings method in the sense that it will detect complex polysaccharides. The Anthrone method has been used throughout this investigation; where Fehlings estimations have been undertaken they are stated as such.

3.3 Chromatography of carbohydrates (Block, R. J. et al. 1953)

The method of descending paper chromatography was used, using Whatman No. 4 paper 18" x 24" and a solvent system of n-butanol, acetic acid and distilled water in a ratio of 4 : 1 : 5 respectively. This mixture separates into two layers, the bottom layer of n-butanol and acetic acid in water was run off onto the floor of the chromatography tank. The top layer, water in n-butanol was run off into the glass trough containing the front of the chromatogram carrying the samples to be separated and identified. The chromatogram was allowed to run for 36 - 48 hours, it was then removed from the tank and developed by immersing in the following mixture: 0.5 g benzidine in 5 ml of glacial acetic acid added to a solution of 90 ml of acetone, 5 ml water and 4 g trichloroacetic acid. The chromatogram was then dried in an oven at 105°F until development was complete.

The standard carbohydrates used in the chromatography were glucose, fructose, sucrose and maltose. The standards and the unknown were applied to the chromatograms by use of a calibrated "Agla" syringe attached to a micrometer. The "Agla" syringe was calibrated by noting the number of turns of the micrometer adjuster required to expel various size drops of distilled water onto the balance pan of a microbalance. Each individual drop was weighed and from the relationship that 1 gm of pure distilled water is equivalent to 1 ml the syringe was calibrated.

When quantitative chromatographic analysis was carried out on the carob extract the standard Anthrone method for carbohydrate estimations was used (Dimler, R. J. et al. 1952) and calibration curves for the individual carbohydrates were constructed.

Section 4

Primary Selection of Fungi

4.1 Primary Selection Criteria

(i) Kjeldahl protein not less than 30%. This is approximately the lower limit of Kjeldahl protein to be nutritionally useful.

(ii) Protein extract not less than 70% of Kjeldahl protein. This provides an allowance of 30% for non-protein nitrogenous matter and loss in extraction.

(iii) Toxicity. Non-toxic for nutritional purposes.

(iv) Growth rate. Not longer than 7 days to reach maximum growth, unless the final yield is the result of a dynamic growth rate.

(v) Protein/carbohydrate ratio. Not less than 0.25 to be economically viable.

(vi) Calorific value not less than 5 Calories per gram, based on dry mycelium. Since on average the fungal mycelium contains 30% Kjeldahl protein, of which an arbitrary figure of 70% is taken as actual protein.

$\therefore \frac{70}{100} \times 30 = 21\%$ actual protein.

$\therefore \frac{21}{100} \times 5 = 1.05$ Cals due to protein.

Since one gram of protein is equivalent to 4 Cals

$\therefore \frac{1.05}{4} = 0.2625$ gram of protein present in one gram of mycelium, or 26.25% protein.

Naturally the fungi selected do not comply with all the criteria, since work in this field is limited and somewhat varied, especially where a variety of media has been used. Some of the fungi selected have a high growth rate but a low protein content or a low growth rate with a high protein content; these fungi have been selected with the intention of attempting to increase their protein content or rate of growth respectively. Thus the above criteria have been applied with a greater flexibility than they reflect, for each criterion has been considered in relation to the other criteria. None of the fungi have been condemned or reprieved in the selection on one criterion alone, except on the criterion of toxicity.

Primary Selected Fungi

4.2 Basidiomycetes

	Crude Protein
Agaricus campestris	62
Agaricus perfuscus copel	50

(Robinson, R. F. 1952) No information on culture supplier was given.

Media : 1.0% Malt Extract

Organism	Original Culture No.	7 day mycelial weight in mg.	Max. mycelial weight in mg.	Days
Coniophora puteana	(2)	79	132	16
Daeclalea quercina	FP 57075-S	113	126	12
Fomes meliae	FP 50336-R	126	125	8
Fomes subroseus	Snell 11	106	116	10
Polyporus palustris	FP 94152	110	114	8
Polyporus spraguei	FP 14857-S	115	132	10
Polyporus monticola	Mad 575	116	131	12
Polyporus nigra	FP 71118	147	155	10
Polyporus oleraceae	FP 48282-R	123	138	10
Trametis serialis	FP 11977	121	140	10
Fomes geotropus	FP 55521-S	123	161	12
Lentinus tigrinus	Mad 466	100	121	14
Polyporus abietinus	FP 71429-R	153	277	20
Polyporus anceps	FP 58526-R	102	114	8
Polyporus tulipferus 11	Mad 517	130	137	10
Polyporus versicolor	FP 57034-R	118	151	10

(Jennison, M. W.; Newcomb, M. D. & Henderson, R. 1955.)

Culture Collection: U. S. Department of Agriculture, Forest Products Laboratory.

Culture No. 2. Institute de Pesquisas Tecnologicas.

Oraganism	% Protein
Agaricus campestris (II)	62.4
Agaricus Merrillii copel B	46.68
Agaricus perfuscus copel (II)	48.85
Auricularia auricula judae	50.10

(Gilbert, F. A. & Robinson, R. F. 1957.) No information on culture supplier was given.

Organism	Culture No.	gm biotia	% Protein	% Fat	Protein/Fat
Polyporus tulipferus	Mad 517(W)	0.28	40.9	1.8	22
Fomes subroseus	Snell 20(B)	1.4	40.4	6.2	6.5
Lentinus lepideus	Mad 534(B)	1.36	36.0	5.7	6.3
Lenzites trabrea	Mad 539(B)	1.2	34.2	5.5	6.2
Poria monticola	Mad 575(B)	2.32	38.9	5.5	7.0
Poria xantha	FP192-Sporo(B)	1.28	38.6	2.3	12.4

(Jennison, M. W.; Richberg, C. G. 1957.)

Culture Collection: U. S. Department of Agriculture, Forest Products Laboratory.

Media Molasses

Organism	% Protein	% Fat
Trichoderma nudum NRRL 2371	54	1.8
Agaricus campestris (L) Fr. CBS	51	0.9
Cantharellus cibarius NRRL 2370	45	2.3

(Reusser, F., Spencer, J. F. T. & Sallans, H. R. 1958.)

Culture Collection: U. S. Department of Agriculture, Northern Utilization Research and Development Division.

4.3 Fungi Imperfecti

Organism	Culture No.	mg/flask	Duration	Kjeldahl Protein	% Protein Extract	E.C.
Rhizoctonia	59	714	4 days	13.4	11.1	1.31
Pestalotia	1-27	618	4 days	19	15	1.51
Pestalotia	1-174	608	4 days	27	27.9	1.53
Pestalotia	1-13	590	4 days	-	-	1.58
Macrophomina	1-145	586	4 days	17	16.9	1.58
Curvularia	1-4	583	4 days	25	20.4	1.55
Hormiactella	1-92	565	4 days	31	25.2	1.65
Hormodendrum	1-154	540	4 days	17	24.2	1.73
Conytrichum	1-39	522	4 days	23	27.2	1.79
Cladosporium	1-83	505	4 days	22.7	12.3	1.84
Phoma	1-14	484	4 days	30	32	1.92

E.C. = Economic coefficient unit, weight of carbohydrate required to produce unit weight of mycelium. (Economic coefficients all less than 2). (Gray, W. D. 1964).

Culture Collection: Ohio State University.

Organism	Culture No.	Crude Protein
Fungi Imperfecti	I-29	37.81

Helminthosporium

Pam, King & Bakke (C.M.I.)

Gray, W. D. (1966).

Culture Collection: Ohio State University.

Organism	k cals/gm. dry mycelium	Culture No.
Mycogone	5.0	I-103
Cylindrocarpon	5.0	I-98

Organism	K cals/gm. dry mycelium	Culture No.
Cephalosporium	5.0	1-87
Nigrosporium	5.0	1-66
Scopularia	5.1	1-95
Dendrophoma	5.2	1-57
Myriothecium	5.3	1-180
Cylindrocephalum	5.3	1-165
Tricothecium	5.3	1-24
Geomyces	5.6	1-155

(Gray, W. D. 1967.)

Culture Collection: Ohio State University.

The fungi primarily selected were compared with those fungi known to be implicated in mycotoxicoses (Borker, E. et al 1966.) and also those fungi whose crude mycelium is known to be toxic to animals (Wright, D. E. 1969.); thus any of those fungi found to be present in the primary selection were excluded from further considerations for protein production.

Fungi obtained for Primary Screening

Cultures obtained

4.4 Basidiomycetes

Organism	Culture No.
Daedalea quercina	38
Polyporus palustris	307A
Polyporus spranguei	227
Trametes serialis	107C
Lentinus tigrinus	68
Polyporus tulipferus	191
Fomes subroseus	
Lentinus lepideus	
Lentinus trabrea	
Poria monticola	
Poria xanthea	
Polyporus anceps	

Cultures obtained from the Forest Products Research Laboratory culture collection, England.

Organism	Culture No.
Fomes meliae (Polyporus meliae)	FP 50336-R
Polyporus nigra (Poria nigra)	FP 71118
Polyporus oleraceae (Poria oleraceae)	FP 48282
Polyporus abietinus (Hirschioporus)	FP 71429-R
Polyporus versicolor	FP R-105
Poria latemarginata (Fomes gestropus)	FP 55521-S

Organism	Culture No.
Polyporus monticola (Poria placenta)	Mad 575

Cultures obtained from the Forest Disease Laboratory culture collection, U. S. A.

4.5 Fungi Imperfecti

Organism	Culture No.
Cochiobolus sativus (Helminthosporium)	75633
Cladosporium cladosporoides	Culture Collection: Ohio State University.

Cultures obtained from the Commonwealth Mycological Institute culture collection.

Organism	Culture No.
Cantharellus	2370
Tricholoma nudum (Fusarium sp.)	2371

Cultures obtained from Northern Utilization Research and Development culture collection, U. S. A.

Organism	Culture No.
Mycogone	I-103
Cylinrocarpon	I- 98
Cephalosporium	I- 87
Scopularia	I- 95
Dendrophoma	I- 57
Trichothecium	I- 24
Geomyces	I-155
Rhizoctonia	I- 59
Pestalotia	I- 27
Hormiactella	I- 92
Hormodendrum	I-154

Cultures obtained from Northern Illinois University culture collection, U. S. A.

Organism	Culture No.
Spicaria elegans	I - 134

Culture obtained from Tate & Lyle Limited. (Original obtained from Northern Illinois University culture collection.)

Organism	Culture No.
Curvularia sp.	M 103
Curvularia sp.	M 104

Cultures obtained from Tate & Lyle Limited.

None of the 54 fungi primarily selected are known to be implicated in mycotoxicoses, although some species of some of the fungi primarily selected are implicated in mycotoxicoses; these are species of Helminthosporium, Cladosporium and Fusarium.

Only 37 of the 54 fungi primarily selected could be obtained, of the 37 fungi obtained 19 were Basidiomycetes and 18 Fungi Imperfecti.

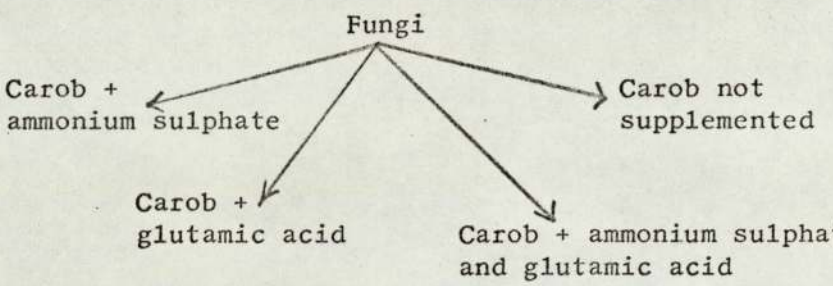
Section 5

Primary Screening and Secondary Selection of Fungi.

Primary Screening and Secondary Selection of Fungi

5.1 Experimental design and media supplementation

Since ammonium sulphate and glutamic acid were the nitrogenous compounds used to supplement the carob media, each fungus was grown on three types of carob supplemented media and an unsupplemented carob medium.



The C:N ratio in the supplemented media was adjusted to 12 - 13 by the addition of the appropriate amount of ammonium sulphate or glutamic acid. In the carob medium supplemented with ammonium sulphate and glutamic acid, each nitrogenous compound contributed half the available nitrogen, C:N ratio 12 - 13.

The two media containing glutamic acid, (i) carob + glutamic acid, (ii) carob + glutamic acid and ammonium sulphate, required the addition of sodium hydroxide, to convert some of the less soluble glutamic acid into the more soluble sodium glutamate. The glutamic acid was first added to the bulk of the media and the whole heated to 60°C. Then 40% sodium hydroxide was added drop wise until the remaining glutamic acid was taken up into solution.

The resulting pH of the media was as follows: (i) carob + glutamic acid, pH 5.25, (ii) carob +glutamic acid + ammonium sulphate, pH 5.3, (iii) carob + ammonium sulphate, pH 4.8, (iv) carob unsupplemented, pH 4.8. The pH of the unsupplemented carob was adjusted by the addition of 40% sodium hydroxide. Since the optimum pH for fungal growth is between pH 4 - 5 (see page 11.) the small difference in pH between the various media will have little if any effect on the resulting fungal growth.

Materials and Methods

5.2 Preparation of Carob Extract

An aqueous extract of the Cypriot carob bean Ceratonia siliqua supplied by Tate & Lyle Limited, was made by immersing approximately 2 - 3 Kg of the kibbled carob, that is the broken pods from which the beans have been removed, in 4 - 5 l of tap water in a stainless steel bucket; the kibbled carob being retained in a muslin bag within the bucket. The bucket and its contents were then autoclaved for 20 minutes at 4 psi. to soften the carob pods and allow the water to leach out the carbohydrates. The resulting liquor was poured off and the kibbled carob sparged with water at 60 - 65°C, to give a total volume of approximately 10.0l of carob extract which was then autoclaved for 15 minutes at 15 psi.

Chromatographic analysis of the carbohydrates in the resultant carob extract did not differ from the results obtained by workers at the Tate & Lyle laboratory, (Scarr, M. P. et al. 1967) that is glucose, sucrose, fructose, primverose and ceretose were present in the carob extract. However primverose and ceretose had previously been identified as maltose at the Tate and Lyle laboratory. Since primverose and ceretose were not available as standards to the author maltose was used.

5.3 Preparation of Race Plates

The race plates were made by pouring 25 ml of autoclaved (15 minutes at 15 psi.) carob extract containing 5 g% total carbohydrates and 2 g% agar at 60°C into 90 mm petri dishes. The carob agar mixture was allowed to solidify and the race plates inspected for infection over a period of 3 days before being used. If the plates were infection free 8 mm diameter appertures were cut into the centre of the solidified carob agar mixture by means of an 8 mm diameter cork borer attached to a 10 ml syringe; this

facilitated rapid removal of the 8 mm diameter piece of solidified carob agar mixture. By using this method a large number of race plates were prepared in a short time.

5.4 Measurement of radial growth of fungi

The maximum and minimum diameter of these transplants were measured by means of vernier calipers every 20 - 24 hours unless the growth rate of the fungus dictated otherwise. Since the race plates were in triplicate the average of the mean diameter in mm was noted and converted to a radius in cm. The square of the radius was then plotted against the time taken to reach that radius.

All fungi to be transplanted into the 8 mm diameter appertures on the race plates were first grown on 25 ml of 5 g% malt extract and 2 g% agar in 90 mm diameter petri dishes, thus establishing a common starting point. When the fungal colony had established itself on the malt extract agar mixture, 8 mm diameter transplants from the perimiter of the fungal colony were taken, (therefore actively growing mycelia was removed) by means of the 8 mm diameter cork borer and syringe. These transplants were then deposited one per race plate into the 8 mm diameter appertures cut in the carob agar mixture.

All aseptic techniques as far as possible were carried out in a positive pressurised hood designed and built by the author, the positive pressure being maintained by sterile air. The use of the hood together with sealing the race plates containing the fungal cultures in polythene bags within the incubator, reduced the infection rate from 15 - 20% to 2 - 5%$\frac{1}{2}$.

5.5 Definitions

The observations made of colony growth were: radial, thin, thick, flat, domed, bushy, diffuse, dense and sparse; these are defined as follows:

Thin or thick, this describes the depth of mycelia over the surface of the carob media.

Flat or domed, this describes the overall shape of the mycelia over the surface of the carob media.

Bushy, this also describes the overall shape of the mycelia over the surface of the carob media.

Diffuse or dense, this describes the distribution of mycelia over the surface of the carob media.

Sparse, this describes limited colony growth in which the circular perimeter is not well defined due to irregular growth at the colonies perimeter.

Results

5.6 Primary Screening

The mean average radius of growth attained by each fungus on the supplemented and unsupplemented carob media was expressed graphically (see Theoretical Considerations, Section 2) and the type of colony growth recorded. (see appendixes). The highest recorded growth rates of the fungi (Basidiomycetes and Fungi Imperfecti) were expressed graphically, and the nitrogen supplements in the carob media supporting the highest recorded growth rates were noted.

Those fungi selected in terms of their radial growth rates for secondary screening were statistically analysed, in terms of their fungal colony diameters attained on the three types of supplemented carob media. This was to ascertain whether or not their difference in radial growth, if any, on the three types of

supplemented carob media were due to chance events or media supplementation, since all other parameters were the same for each fungus. The type of growth of these fungi on the supplemented carob media was also taken into account. It is these observations and statistical results of the fungi selected for secondary screening in terms of their radial growth that are now presented.

Basidiomycetes

Poria latemarginata

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	23	1.21
	47	6.40
	54	8.50
	71	15.10
	75	17.64

Polyporus palustris

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	17	0.16
	43	1.17
	89	6.25
	115	10.89
	138	17.22

Polyporus meliae

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	18	0.40
	43	1.10
	90	5.66
	115	9.18
	138	14.44

Polyporus anceps

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	17	0.16
	43	1.16
	90	4.75
	115	7.64
	138	10.99

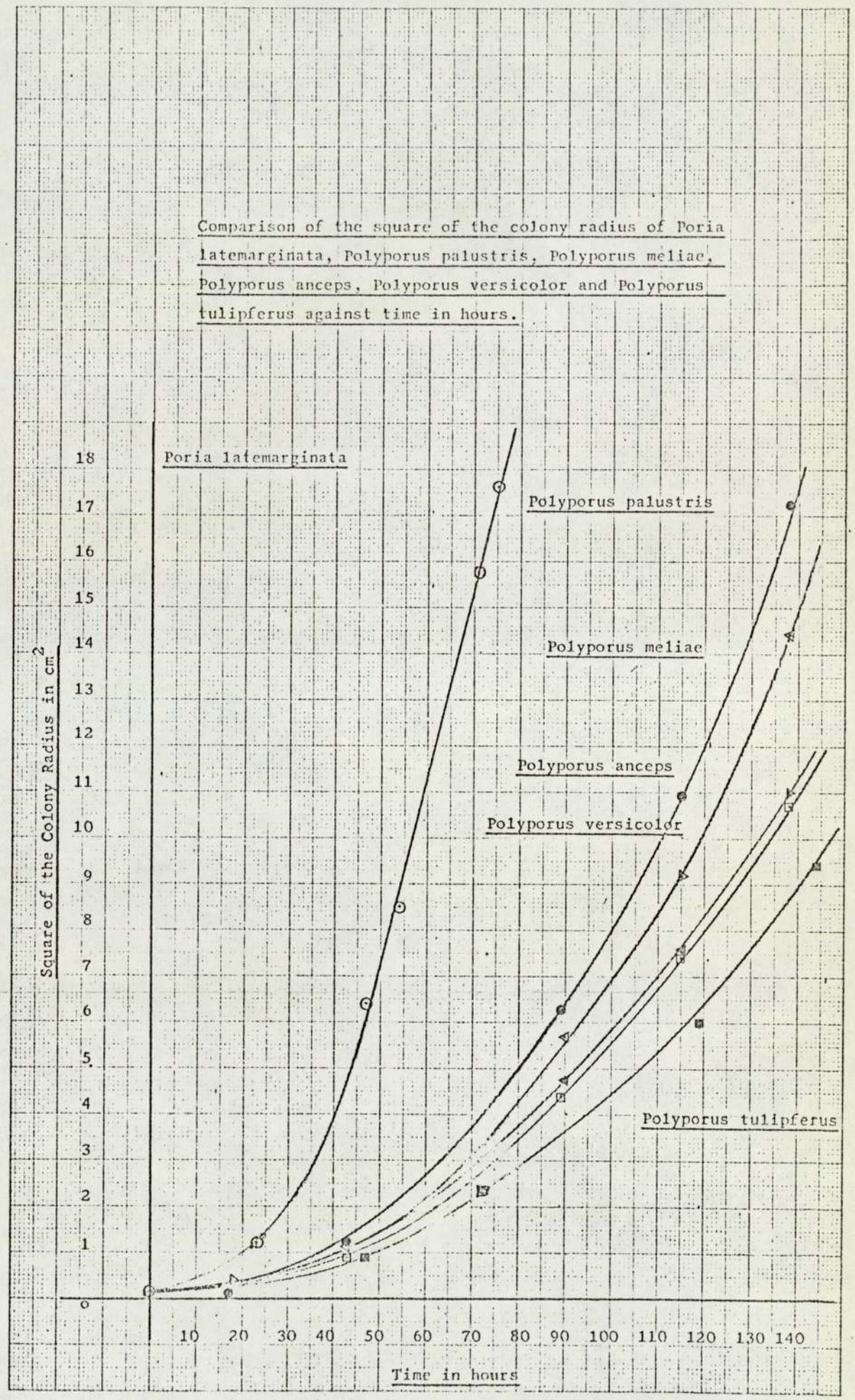
Polyporus versicolor

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	18	0.30
	43	0.90
	89	4.40
	115	7.37
	138	10.75

Polyporus tulipferus

Nitrogen supplements	Time	R^2cm
Glutamic acid	0	0.16
	23	0.16
	47	0.90
	72	2.19
	119	5.9
	144	5.39

The Basidiomycetes formed three seperate spacial groups. Group (1)
aining those fungi with maximum radial growth rates; Group (3) those
i with minimal radial growth rates; Group (2) contained those fungi
intermediate radial growth rates. For the sake of clarity only groups
and (2) have been graphically presented, for it was those fungi in
e groups which were selected for secondary screening.


Comparison of the square of the colony radius of Poria latemarginata, Polyporus palustris, Polyporus meliae, Polyporus anceps, Polyporus versicolor and Polyporus tulipferus against time in hours.
Poria latemarginata
Polyporus palustris
Polyporus meliae
Polyporus anceps
Polyporus versicolor
Polyporus tulipferus
Square of the Colony Radius in cm²
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Time in hours

Statistical Analysis ' t ' Test

Basidiomycetes

Poria latemarginata

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	77	AS	80	GA + AS	84
	77		80		82
	73		72		86
means	75.6		77.3		84
Colony growth :	Thick		Thick		Thick
	Flat		Flat		Flat
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between the radial growth on the ammonium sulphate and glutamic acid supplemented carob media was not significant.

$$' t ' = 2.2$$

$$\infty_F = 4$$

$$p > 0.05$$

The difference between the radial growth on the ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media was very highly significant.

$$' t ' = 17$$

$$\infty_F = 4$$

$$p < 0.001$$

Since there was no significant difference between ammonium sulphate and glutamic acid supplemented carob media, but a very highly significant difference between ammonium sulphate and

ammonium sulphate plus glutamic acid supplemented carob media, there will also be a very highly significant difference between the latter and the glutamic acid supplemented carob media.

Therefore the greater radial growth of Poria latemarginata may be attributed to the carob media containing glutamic acid and ammonium sulphate. This corresponded with the observations of the colony growth on this carob media.

Polyporus tulipferus

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	71	AS	58	GA + AS	73
	72		63		74
	70		60		56
means	71		60.3		67.6
Colony growth :	Thick		Thin		Thick
	Flat		Flat		Flat
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between the radial growth on the ammonium sulphate and glutamic acid and the ammonium sulphate supplemented carob media was significant.

$$\text{'t'} = 7.7$$

$$\infty_F = 4$$

$$p > 0.001 < 0.01$$

The difference between the radial growth on the ammonium sulphate and glutamic acid supplemented carob media was highly significant.

$$\text{'t'} = 5.5$$

$$\infty_F = 4$$

$$p < 0.01 > 0.001$$

Therefore the greater radial growth of Polyporus tulipferus may be attributed to the carob media containing glutamic acid. This also corresponded with the observations of colony growth on this carob media.

Polyporus anceps

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	57	AS	62	GA + AS	67
	58		60		66
	57		65		66
means	57.3		62.3		66.3
Colony growth :	Thin		Thick		Thick
	Flat		Flat		Flat
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between the radial growth on the ammonium sulphate and ammonium sulphate plus glutamic acid supplemented carob media was highly significant.

$$t = 7$$

$$\omega_F = 4$$

$$p > 0.001 < 0.01$$

The difference between the radial growth on the glutamic acid and ammonium sulphate plus glutamic acid supplemented carob media was very highly significant.

$$t = 18$$

$$\omega_F = 4$$

$$p < 0.001$$

Therefore the greater radial growth of Polyporus anceps may be attributed to the carob media containing glutamic acid and ammonium sulphate. This also corresponded with the observations of colony growth on this carob media.

Polyporus versicolor

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	57	AS	61	GA + AS	65
	61		61		66
	57		63		66
means	58.3		61.6		65.6
Colony growth :	Thin		Thick		Thick
	Flat		Flat		Flat
	Sparse		Dense		Dense
	Radial		Radial		Radial

The difference between the radial growth on the ammonium sulphate and the glutamic acid plus ammonium sulphate supplemented carob media was significant.

$$'t' = 3$$

$$\infty_F = 4$$

$$p > 0.001 < 0.01$$

The difference between the radial growth on the glutamic acid and the glutamic acid plus ammonium sulphate supplemented carob media was highly significant.

$$'t' = 6$$

$$\infty_F = 4$$

$$p > 0.001 < 0.01$$

The greater radial growth may have been attributed to the carob media containing glutamic acid and ammonium sulphate. This also corresponded with the observations of colony growth on this media.

Polyporus palustris

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	45	AS	86	GA + AS	87
	42		73		82
	71		82		80
means	52.6		80.3		80.3
Colony growth :	Thick		Thick		Thick
	Flat		Flat		Flat
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between the radial growth on the ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media was highly significant.

$$' t ' = 7$$

$$\infty_F = 4$$

$$p > 0.001 < 0.01$$

The difference between the radial growth on the glutamic acid and glutamic acid plus ammonium sulphate supplemented carob media was very highly significant.

$$' t ' = 31$$

$$\infty_F = 4$$

$$p > 0.001 < 0.01$$

The greater radial growth may have been attributed to the carob media containing glutamic acid and ammonium sulphate. This also corresponded with the observations of colony growth on this carob media.

Polyporus meliae

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	65	AS	70	GA + AS	75
	65		76		77
	71		78		76
means	67		72		76
Colony growth:	Thin		Thin		Thick
	Flat		Flat		Flat
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between the radial growth on ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media was not significant.

$$'t' = 1.2$$

$$\infty_F = 4$$

$$p > 0.1$$

The difference between the radial growth on the glutamic acid and glutamic acid plus ammonium sulphate supplemented carob media was very highly significant.

$$'t' = 45$$

$$\infty_F = 4$$

$$p > 0.001 < 0.01$$

Since there was no significant difference in radial growth on the ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media; a very highly significant difference between radial growth on glutamic acid and glutamic acid plus ammonium sulphate supplemented carob media, it followed that there

would be a very highly significant difference in radial growth between glutamic acid and ammonium sulphate supplemented carob media. However, since observations of colony growth favoured the selection of the media containing glutamic acid plus ammonium sulphate this media was selected.

The following Basidiomycetes, Fomes sub-roseus, Daedalea queraisa and Coniophora failed to grow sufficiently on malt extract over a six day period in petri dishes to enable transplants to be taken for race plate analysis. Therefore fragments of the fungal colonies were transplanted onto the supplemented and unsupplemented carob media in petri dishes in an attempt to obtain sufficient mycelia for race plate analysis.

Results after a six day period.

Coniophoria : limited centre growth on supplemented and unsupplemented carob media.

Fomes sub-roseus : limited centre growth on supplemented and unsupplemented carob media.

Daedalea queraisa : failed to grow on supplemented and unsupplemented carob media.

These fungi were then excluded from further consideration.

Fungi Imperfecti

Mycogone I 103

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	21	0.30
	43	1.14
	67	2.13
	92	5.29
	115	7.02

Dendrophoma I 57

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	21	0.33
	42	0.96
	67	2.16
	92	2.62
	115	3.84

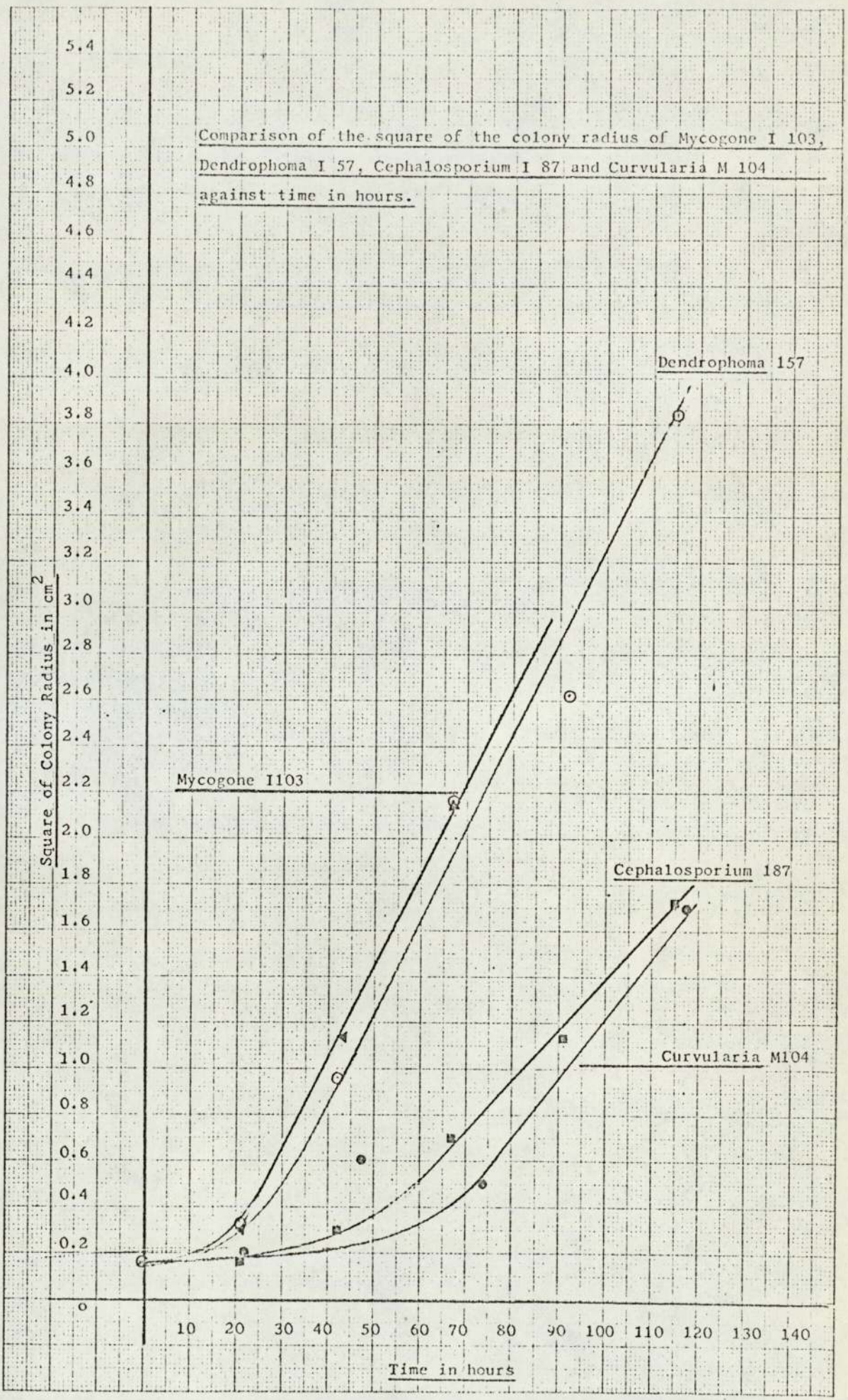
Cephalosporium I 87

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	21	0.16
	42	0.25
	67	0.69
	91	1.13
	115	1.72

Curvularia M 104

Nitrogen supplements	Time	R^2cm
Glutamic acid +	0	0.16
Ammonium sulphate	22	0.20
	47	0.46
	74	0.92
	117	1.36

The Fungi Imperfecti formed two separate spacial groups, two fungi
group. Since this was the total number of Fungi Imperfecti whose
al growth could be measured, they were selected for secondary
ening sincer there was not a third group to act as a reference point
n the Basidiomycetes.


Comparison of the square of the colony radius of Mycogone I 103, Dendrophoma I 57, Cephalosporium I 87 and Curvularia M 104 against time in hours.
Square of Colony Radius in cm²
5.4
5.2
5.0
4.8
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Dendrophoma 157
Mycogone I103
Cephalosporium 187
Curvularia M104
10 20 30 40 50 60 70 80 90 100 110 120 130 140
Time in hours

Statistical Analysis ' t ' Test

Fungi Imperfecti

Dendrophoma I 57

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	30	AS	28	GA +AS	48
	50		23		30
	36		23		40
means	38.6		24.6		39.3
Colony growth:	Thick		Thin		Thick
	Domed		Domed		Domed
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between radial growth on the glutamic acid and glutamic acid plus ammonium sulphate supplemented carob media was significant.

$$' t ' = 3.3$$

$$^{\infty}F = 4$$

$$p < 0.1$$

The difference between radial growth on the ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media was very highly significant.

$$' t ' = 134$$

$$^{\infty}F = 4$$

$$p < 0.001$$

The greater radial growth may have been attributed to the carob media containing glutamic acid and ammonium sulphate. The observations of colony growth on this media also corresponded with the above results.

Mycogone I 103

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	47	AS	22	GA + AS	53
	52		27		overgrown
	25		32		overgrown
means	41.3		27		53
Colony growth :	Thick		Thin		Thick
	Domed		Domed		Domed
	Dense		Dense		Dense
	Radial		Radial		Radial

The difference between radial growth on glutamic acid and glutamic acid plus ammonium sulphate supplemented carod media was very significant.

$$t = 15$$

$$\infty_F = 2$$

$$p < 0.01 > 0.001$$

Therefore the difference between radial growth on ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media would obviously be significant.

The greater radial growth may therefore have been attributed to the carob media supplemented with glutamic acid and ammonium sulphate. The observations of colony growth on this media also corresponded with the statistical results.

Cephalosporium I 87

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	23	AS	13	GA + AS	25
	21		12		28
	23		13		26
means	22.3		12.6		26.3
Colony growth :	Thin		Thin		Thin
	Flat		Flat		Flat
	Dense		Limited		Dense
	Radial		Radial		Radial

The difference between radial growth on glutamic acid and glutamic acid plus ammnoium sulphate supplemented carob media was very significant.

$$'t' = 6.6$$

$$^{\infty}F = 4$$

$$p < 0.01 > 0.001$$

Therefore it was obvious that the difference between ammonium sulphate and glutamic acid plus ammonium sulphate would also be significant.

The greater radial growth may have been attributed to the carob media supplemented with glutamic acid and ammonium sulphate. This also corresponded with the observations on colony growth.

Curvularia M 104

Final diameter of colonies in mm.

Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm	Nitrogen Supplement	Dia. mm
GA	26	AS	18	GA + AS	23
	overgrown		21		24
	overgrown		22		overgrown
means	26		20.5		23.5
Colony growth:	Thin		Thin		Thick
	Flat		Flat		Flat
	Sparse		Sparse		Sparse
	Radial		Radial		Radial

The difference between radial growth on glutamic acid and glutamic acid plus ammonium sulphate supplemented carob media was not significant.

$$* t * = 2.0$$

$$\infty_F = 1$$

$$p > 0.1$$

The difference between radial growth on ammonium sulphate and glutamic acid plus ammonium sulphate supplemented carob media was not significant.

$$* t * = 4.5$$

$$\infty_F = 3$$

$$p = 0.02$$

The difference between radial growth on ammonium sulphate and glutamic acid supplemented carob media was not significant.

$$* t * = 7$$

$$\infty_F = 2$$

$$p < 0.02 > 0.01$$

Therefore the greater radial growth could not be attributed to media supplementation, and must have been due to chance. However from the observations of colony growth the media supplement that was selected to be used in the secondary screening of Curvularia M 104 was glutamic acid plus ammonium sulphate.

The following Fungi Imperfecti failed to grow sufficiently on malt extract over a six day period in petri dishes to enable transplants to be taken for race plate analysis.

Curvularia M 103

Cochiobolus sativus

Homiactella I 92

Fusarium 2371

Cladosporium cladosporoides I 75

Fragments of the above fungi were then removed from the malt extract, and transplanted onto the supplemented and unsupplemented carob media in an attempt to obtain sufficient mycelia for race plate analysis.

Results after a six day period.

The following fungi failed to grow sufficiently on the supplemented and unsupplemented carob media for transplants to be made for race plate analysis.

Curvularia M 103

Hormiactella I 92

Fusarium 2371

These fungi were excluded from further consideration.

Although Cladosporium cladosporoides failed to produce sufficient mycelia, except for a few secondary colonies on the carob media supplemented with ammonium sulphate or glutamic acid, it did however rapidly colonise the carob media supplemented with glutamic acid and ammonium sulphate. The rate of secondary colony formation

made it impossible for secondary colony counts to be made or measured. A similar situation was found in the case of Cochiololus sativus, however secondary colonies were not produced on the carob media supplemented with glutamic acid plus ammonium sulphate or any of the other supplemented or unsupplemented carob media, but the resultant growth of Cochiobolus sativus on the carob media supplemented with glutamic acid plus ammonium sulphate was sufficient for race plate analysis. However race plate analysis on these two fungi was not carried out since it was obvious from the above results that they were worthy of further consideration, so these two fungi were selected for secondary screening.

Although some Fungi Imperfecti grew sufficiently on malt extract media to allow transplants to be made for race plate analysis, they failed to grow on the supplemented and unsupplemented carob media; these fungi were Trichothecium I 124 and Rhizoctonia I 159, although Rhizoctonia I 159 did attain a colony radius of 0.9 cm (r^2 = 0.81) this was over a duration of 120 hours.

In contrast to this Spicaria elegans was slow to grow on malt extract media and did not produce any secondary colonies, however on the supplemented and unsupplemented carob media secondary colonies were produced. These secondary colonies were produced at such a rate that it was impossible to count them accurately or measure them. By pure observation it appeared that the carob media supplemented with glutamic acid and ammonium sulphate supported a greater number of actively growing secondary colonies compared with the growth on the other supplemented and unsupplemented carob media. Thus on the basis of this observation Spicaria elegans was selected for secondary screening.

Fungi Selected for Secondary Screening

5.9 Basidiomycetes

Organism	Nitrogen Supplements
Poria latemarginata	Glutamic acid + Ammonium sulphate
Polyporus anceps	Glutamic acid + Ammonium sulphate
Polyporus versicolor	Glutamic acid + Ammonium sulphate
Polyporus palustris	Glutamic acid + Ammonium sulphate
Polyporus meliae	Glutamic acid + Ammonium sulphate
Polyporus tulipferus	Glutamic acid

5.9A Fungi Imperfecti

Organism	Nitrogen Supplements
Cladosporium cladosporoides	Glutamic acid + Ammonium sulphate
Spicaria elegans	Glutamic acid + Ammonium sulphate
Mycogone I 103	Glutamic acid + Ammonium sulphate
Dendrophoma I 57	Glutamic acid + Ammonium sulphate
Cochiobolus sativus	Glutamic acid + Ammonium sulphate

Discussion and Conclusions

The Basidiomycetes and Fungi Imperfecti selected for secondary screening, (except for Cladosporium cladosporoides and Spicaria elegans, which were selected independantly) complied with all the criteria used in their selection, that is, (i) radial growth rate, (ii) statistical analysis, (iii) type of colony growth, but consideration of any one of the criteria alone would have also lead to their selection. Therefore those fungi and nitrogen supplements selected for secondary screening satisfy all three criteria equally well.

Section 6

Secondary Screening - Submerged Culture

Experiment 1. Submerged Culture

Experiment 1 - Submerged Culture

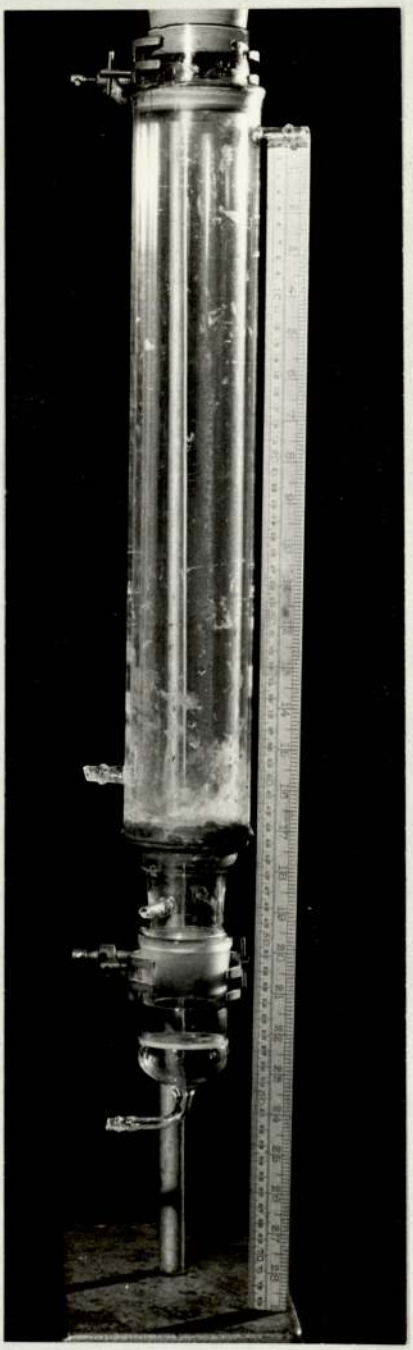
6.1 Experimental Design

In the primary screening of members of the Basidiomycetes and Fungi Imperfecti, the C:N ratio selected was between 12 - 13. This C:N ratio was the mean of the average C:N ratios for fungi used in protein synthesis. If the C:N ratio is adjusted such that the g% carbon concentration is slightly in excess of its optimum concentration; (higher C:N ratio) the rate of utilization of nitrogen may be less, either way possibly showing a preference for carbohydrate uptake. If this is valid, then when the carbohydrate reaches its optimum concentration and the C:N ratio is optimum, the carbohydrate and nitrogen should be utilized at the correct differential rates for growth and protein synthesis; therefore the C:N ratio staying fairly constant throughout the fermentation. It was therefore decided to adjust the C:N ratio from 12 - 13 to 13 - 14, based on this hypothesis for those fungi selected for screening in submerged culture in this Experiment 1sc.

Although the carbohydrate concentrations and nitrogen concentrations have been followed throughout the fermentations, they were observed and used as indications as when to terminate the experiment to obtain as accurately as possible the correct fermentation time and total producable biomass. They were not intended to be used, or interpreted as kinetic studies. It would have been of great value however if the growth kinetics could have been studied of the fungi, for then the experiments could have been terminated as the fungi entered the stationary phase of growth; unfortunately the sample appertures of the 1.0L tower fermenters would not readily allow the passage of samples of media containing fungal mycelium.

Materials and Methods

The secondary screening of those fungi selected for the submerged culture was carried out in 1.0L tower fermenters as shown below. These tower fermenters were sterilized by passing steam through them for 24 hours at atmospheric pressure.



1.0L Tower Fermenter

Plate 1

6.2 Preparation of carob media

The media, aqueous carob extract was not prepared as previously described for the Primary Screening, instead of autoclaving

the kibbled carob and water at 4 p.s.i. for 5 minutes, the whole was free steamed for 5 - 10 minutes at atmospheric pressure; the kibbled carob was then sparged with hot water at 60°C and the resulting extract was then autoclaved at 15 p.s.i. for 15 minutes as before. The resultant liquor was wine red in colour, and all the colloidal and fine particulate matter was precipitated and settled out of solution, therefore giving a translucent wine red liquor. Chromatographically there was no difference, in terms of the type of carbohydrates extracted between this extract and the previous extracts used in the primary screening, except a decrease in the efficiency of extraction of the carbohydrates.

Those fungi that had been selected for secondary screening in submerged culture were first grown on race plates, composed of this clear carob extract and 2 g% agar. Their radial growths were measured and compared with those obtained in the primary screening. It was found that no significant difference existed between the two sets of results; therefore suggesting that the only difference between the two types of media was clarity, most probably due to a reduction of the tannin content of the carob extract.

The pH of the carob media used in the submerged culture experiment was adjusted to the pH of the media used in the primary screening (pH 4.5 - 5.5) by the addition of concentrated hydrochloric acid or 40 g% sodium hydroxide. It was found that when the pH of the carob media containing glutamic acid was adjusted not all of the glutamic acid was in solution. The addition of 40 g% sodium hydroxide converted some of the glutamic acid in solution to monosodium glutamate; this being somewhat more soluble than glutamic acid allowed the remaining glutamic acid to be taken up into solution and the correct pH was obtained.

Since there is present an acid and a weak acidic salt of that acid, (in the carob media containing glutamic acid) it is possible that this buffer system may maintain the pH of the carob media within the optimum pH limits for fungal growth, depending of course on how the acid and acidic salt are utilized by the fungi. Also glutamic acid and ammonium sulphate both contributed half the available nitrogen as in the primary screening.

The temperature of each fermentation was maintained at 30°C as in the primary screening. At the beginning of each fermentation 10 ml of 2 g% aqueous solution of silicon was added to prevent foaming due to the aeration of the carob media.

6.3 Inoculum for 1.0L tower fermenters

Petri dishes containing 25 ml of media (same composition as that used in the 1.0l tower fermenter) plus 2 g% of agar were prepared and then inoculated with spores in the case of the Fungi Imperfecti and mycelium in the case of the Basidiomycetes. The resultant mycelial growth (mycelium and spores in the Fungi Imperfecti) was allowed to completely cover the surface of the media in the petri dish, at which point the entire contents of the petri dish was aseptically transferred to a Waring blender and homogenised for 3 minutes with a proportion of the media to be used in the 1.0l tower fermenter for that particular fungus. In the estimation of dry weights and protein content of the fungal mycelium the amount of agar present in the fermenter was taken into account.

Some of the cultures of fungi prepared in this manner obviously completed their allowed growth at inconvenient times. These cultures were stored in a refrigerator at 4°C until one day before they were required, when they were again transferred to the incubator at 30°C to allow them to equilibrate before being transferred to the 1.0L tower fermenter at the same temperature.

Fungi to be screened

6.4 Basidiomycetes	Nitrogen supplements
Poria latemarginata	GA + AS
Polyporus tulipferus	GA
Polyporus meliae	GA + AS
Polyporus versicolor	GA + AS
Polyporus anceps	GA + AS
Polyporus palustris	GA + AS

6.5 Fungi Imperfecti	Nitrogen supplements
Cladosporium cladosporoides	GA + AS
Spicaria elegans	GA + AS
Mycogone I 103	GA + AS
Dendrophoma I 57	GA + AS
Cochiobolus sativus	GA + AS

6.6 Abbreviations and Definitions

Chemical Name		Abbreviation
Ammonium sulphate	=	AS
Glutamic acid	=	GA

Definitions

Yield, this was defined as the g% dry weight of mycelium produced in a given time divided by the g% weight of carbohydrates utilized. This was therefore an expression of the grams of dry mycelium produced per gram of carbohydrate utilized.

Results

6.7 Basidiomycetes

Organism: Poria latemarginata

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.4. Initial C:N ratio 13.5.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass produced	Yield	% Protein
0	5.3	0.157			
18.75	4.9	0.148			
42.25	4.3	0.147			
66.25	2.8	0.089		0.23	
91.00	6.1	0.065			
99.25	5.6	0.060			
115.00	4.5	0.061			
121.00	3.5	0.060	0.58	0.32	44.01

Sample of media at the end of the fermentation was analysed by the Fehlings method for carbohydrates which gave the following result: Sample hydrolysed = 3.6 g% Carbohydrates.

The type of growth of Poria latemarginata attained was not a mass of entangled mycelium as might be expected, but spherical hollow pellets, the size varying between 1 - 3 cm. From the surface of these pellets were spikey projections of various sizes, but not exceeding the diameter of the pellet, this giving the appearance of a spiked mace.

As can be observed from the above table the carbohydrate content of the media at 66.25 hours was 2.8 g%, at 91.0 hours it had risen, according to the anthrone estimation, to 6.1 g% and then proceeded to decrease, and at 121.0 hours reached 3.5 g%. The Fehlings method for total carbohydrates estimation on this final sample was 3.6 g%. ($\pm$ 0.1 which can be attributed to experimental error). It may have been that some reducing metabolite was excreted into the media by Poria latemarginata and was then re-utilized. Throughout the latter part of the fermentation a strong smell that can only be described as an alcoholic-ester smell persisted.

The nitrogen content decreased from 0.089 g% at 66.25 hours to

an average constant of 0.061 g% throughout the remainder of the fermentation. It is possible that these phenomena could have been due to a nitrogen deficiency in the media from 66.25 hours onward, which also corresponds with the first minimum estimation of carbohydrate.

Organism: Polyporus tulipferus

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.4. Initial C:N ratio 12.9.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass produced	Yield	% Protein
0	5.0	0.154			
17.5	4.7	0.148			
41.00	4.3	0.144			
60.5	4.0	0.130			
89.75	4.4	0.070			
99.00	3.4	0.058			
114.00	1.8	0.047			
121.00	2.6	0.047	0.55	0.23	51.75

Sample of media at the end of the fermentation analysed by the Fehlings method for carbohydrates gave the following result:

Sample hydrolysed = 2.8 g% carbohydrates.

The type of growth Polyporus tulipferus attained was small, hollow spherical pellets measuring between 0.2 - 1.0 cm in diameter. From each pellet what appeared to be a hyphal thread approximately 3 x their diameter projected through the surface of the pellet.

When the carbohydrate and nitrogen estimations relating to Polyporus tulipferus were compared with those of Poria latemarginata, it was found that they followed a similar pattern. There was also the accompanying alcoholic-ester smell as in the case of Poria latemarginata. The same explanation given for Poria latemarginata may have also been applicable in this case.

The submerged culture experiments on the following Basidiomycetes were terminated after 70 - 90 hours since no measurable growth or utilization of nutrients had occurred.

Polyporus meliae

Polyporus versicolor

Polyporus anceps

Polyporus palustris

6.8 Fungi Imperfecti

Organism: Cochiobolus sativus

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.3. Initial C:N ratio 13.8.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass produced	Yield	% Protein
0	5.2	0.151			
16.75	5.0	0.146			
40.75	4.8	0.151			
64.75	4.8	0.151	0.51	1.2	35.35

The type of growth attained by Cochiobolus sativus was small, hollow, spherical pellets approximately 2 - 4 mm in diameter.

The results indicated that Cochiobolus sativus was most efficient, producing 1.2 grams of mycelium per gram of carbohydrate, however this was quite impossible since it suggested that all the carbohydrates utilized were utilized for mycelial growth and none for basal metabolism. The result would have been plausible if there had been a sharp decrease in the nitrogen content of the media, for the fungus may have then obtained sufficient energy for basal metabolism by the catabolism of glutamic acid, but as can be observed from the results the nitrogen in the media was not utilized.

This discrepancy may have been due to the fungus excreting a reducing nitrogenous metabolite into the media.

Organism: Cladosporium cladosporoides

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.2. Initial C:N ratio 14.5.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass produced	Yield	% Protein
0	5.5	0.151			
17.6	4.4	0.109			
* 24.00	3.7	0.100			
41.6	3.3	0.100	0.74	0.34	31.87

* Aeration increased to 600 ml/min to prevent scinter clogging.

The type of growth attained by Cladosporium cladosporoides was small, compact pellets, the shape of rice grains, these were approximately 2 - 3 mm long.

Organism: Spicaria elegans

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.3. Initial C:N ratio 13.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass produced	Yield	% Protein
0	5.5	0.168			
13.8	5.2	0.140			
* 21.96	4.1	0.140			
24.00	3.9	0.134			
40.3	3.2	0.112	1.10	0.48	29.78

* Scinter clogging, aeration increased to 570 ml/min N.T.P.

The type of growth attained by Spicaria elegans was similar to that of Cladosporium cladosporoides, that is, compact rice shaped pellets 2 - 4 mm long.

Organism: Mycogone I 103

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.2. Initial C:N ratio 14.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass produced	Yield	% Protein
0	5.5	0.151			
16.6	4.5	0.132			
* 22.9	3.8	0.115			
41.9	2.1		0.70	0.20	45.9

* Aeration increased to 600 ml/min N.T.P. to releave clogging of scinter.

The type of growth attained by Mycogone I 103 was small, hollow, spherical pellets approximately 2 - 5 mm in diameter.

Organism: Dendrophoma I 57

Temperature 30°C; Aeration 410 ml/min N.T.P. Pitch volume 1.01.

Initial pH 5.4. Initial C:N ratio 13.5.

Time in hours	g% Carbohydrates	g% Nitrogen	g% Biomass production	Yield	% Protein
0	5.5	0.168			
15.00	4.9	0.158			
42.00	3.5	0.131	0.26	0.13	42.36

The type of growth attained by Dendrophoma I 57 was small, irregular shaped hollow pellets varying in size from 2 - 5 mm.

6.9 Selection of Fungi for further analysis

The selection of fungi was based on the following criteria:

(i) Fermentation time

(ii) g% Biomass produced

(iii) g% Protein

(iv) Yield

(v) Nitrogen utilized

Organism	Time in hours	g% Biomass	Yield	g% Protein	g% Nitrogen utilized
Basidiomycetes					
Poria latemarginata	121	0.58	0.32	44.10	0.096
Polyporus tulipferus	121	0.55	0.23	51.75	0.107
Fungi Imperfecti					
Cladosporium cladosporoides	41.6	0.74	0.34	31.87	0.061
Spicaria elegans	40.3	1.10	0.48	29.78	0.056
Mycogone	41.9	0.70	0.20	45.90	
Dendrophoma	42.0	0.26	0.13	42.76	0.037
Cochiobolus sativus	64.75	0.51	1.2	35.35	0.00

table 1

Discussion and Conclusions

Consideration of the resultant values of table 1. of the criteria used in the selection of fungi grown under submerged culture conditions showed that the Basidiomycetes with a fermentation time of 121 hours and a g% Biomass between 0.5 - 0.6 were unsuitable for further investigation at this moment, although their g% protein content was somewhat higher than the Fungi Imperfecti.

Those fungi worthy of further investigation were Spicaria elegans, Cladosporium cladosporoides and Mycogone which were all Fungi Imperfecti. Although their g% protein was somewhat low in

the case of Spicaria elegans and Cladosporium cladosporoides 29.78% and 31.87% respectively. It may be possible however to increase the protein content of these fungi by optimization of the media. The fungi finally selected, Cladosporium cladosporoides, Spicaria elegans and Mycogone have been named AD_1, AD_2 and AD_3 respectively and are now referred to as such throughout this thesis.

Selected fungi AD_1, AD_2 and AD_3.

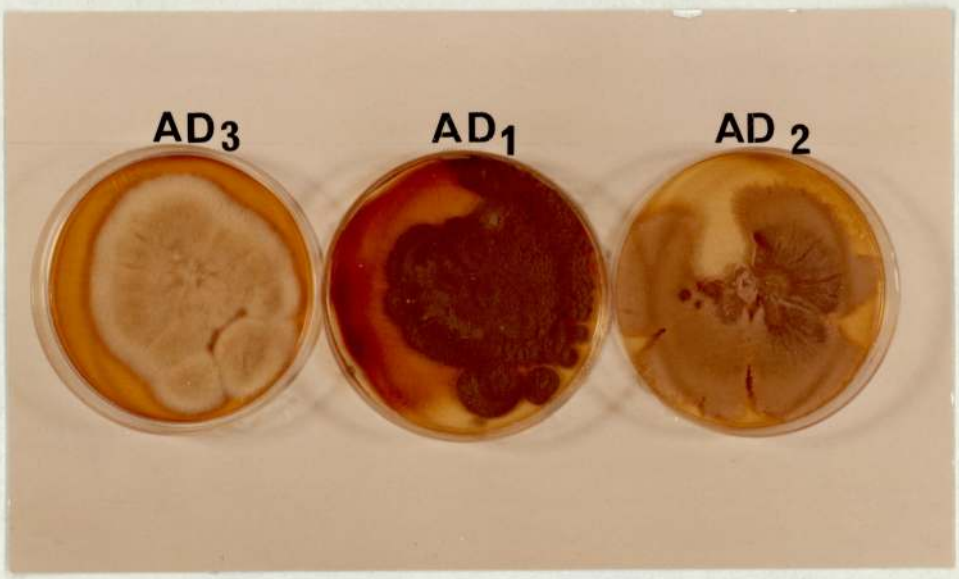


Plate 2

6.9A Changes in carob media C:N ratio during Submerged Culture (Experiment 1sc.) of AD_1, AD_2 and AD_3.

Organism	Time in hours	C:N	
AD_1	0	14.5	
	17.6	16.1	
	24.00	15	average 14.4
	41.6	12	
AD_2	0	13	
	13.8	14.8	
	21.96	11.7	
	24.96	11.6	average 11.5
	40.3	11.4	
AD_3	0	14	
	16.6	13	
	22.9	13	average 13
	41.9	sample lost	

table 2

Conclusion

It can be seen that in the case of AD_2 the C:N ratio stabilized between 11.4 and 11.7, the average of the three results being 11.5 (table 2.). In the case of AD_1 and AD_3 (table 2.), since the results were less static than those of AD_2, although AD_3 did stabilize at a C:N ratio of 13, (but only two results) it was decided to set the C:N ratio at 11.5 in the next submerged culture experiment for AD_1, AD_2 and AD_3.

Section 7

Adjustment of carob media C:N ratio; determination of nitrogen and carbohydrate utilization by AD_1, AD_2 and AD_3.

Experiment 2sc. - Submerged Culture

Experiment 2sc.

Adjustment of carob media C:N ratio; determination of nitrogen and carbohydrate utilization by AD_1, AD_2 and AD_3.

7.1 Experimental design

The following experiments were constructed to see the effect of the approximated optimum C:N ratio of 11.5 obtained from Experiments 1sc., (table 2.) on biomass production and protein synthesis by comparing the results obtained in these experiments (Experiments 2sc.), with those obtained in Experiment 1sc. To ascertain whether or not the Glutamic acid and Ammonium sulphate nitrogen was utilized equally or in some ratio, and if this nitrogen supplementation of the carob media was wholly responsible for protein synthesis; this was achieved by comparing the growth of the fungi in carob media which did not contain any nitrogen supplements. (Control experiment 2sc.) These Control experiments also allowed a comparison of an unbuffered system to be made with the buffered system (Glutamic acid, Monosodium glutamate in supplemented carob media) in terms of pH. Chromatographic investigations were made to ascertain which carbohydrates were utilized, and if sucrose was one of these carbohydrates, whether or not it was first enzymically hydrolised in the carob media before being assimilated by the fungi.

Materials and Methods

The experiments were carried out in 1.0 litre tower fermenters using the same type of carob media preparation as previously described in Experiment 1sc. Total carbohydrate was 4.25 g% and total nitrogen 0.148 - 0.149 g% giving a C:N ratio of 11.5. The nitrogen supplements were glutamic acid and ammonium sulphate in equal proportions in terms of nitrogen concentration. Fermentation temperature was $30^{o}C$. Foaming was again prevented by use of silicone.

The total carbohydrate estimations were carried out as in the previous experiments, except the reducible carbohydrate concentrations were followed by the Fehlings method throughout the fermentation. Quantitative paper chromatography of the carbohydrates as described in the Chemical Analyses Section (3) was carried out on the first and final samples of media in each case to determine the individual carbohydrate concentrations. The nitrogen determinations were made by the Kjeldahl method (Chemical Analyses Section 3.), the Ammonium sulphate nitrogen being determined by direct distillation in the Kjeldahl apparatus. The nitrogen due to the Glutamic acid was then obtained by subtraction of the Ammonium sulphate nitrogen and nitrogen normally present in the carob media from the total nitrogen. The protein estimations of the fungal mycelium were obtained as before (total nitrogen x 6.25).

7.2 Inoculum for 1.0 litre tower fermenter

The inoculum was from liquid surface culture. This was prepared by inoculating 100 ml. of sterile medium in 250 conical flasks with the spores of the appropriate fungi (AD_1, AD_2 and AD_3). The cultures were allowed to grow until the entire surface of the media was covered with a mycelial mat showing spores. The cultures were stored in the refrigerator until one day before they were required, when they were transferred back to the incubator at 30°C. The media was carefully poured off and the mycelial mat and spores homogenised with a known quantity of the media used in the Experiment 2sc. All media losses in the transfers were taken into account in the final pitching volume in the tower fermenter.

7.3 Abbreviations

The heading for the results of carbohydrate estimations by the Anthrone method has been abbreviated to 'A'. The heading for the

results of the reducible carbohydrates by the Fehlings method has been abbreviated to 'F'.

Results

7.4 Fungi Imperfecti

Organism: AD_1.

Temperature 30°C; Aeration 490 ml/min N.T.P. Pitch volume 970 ml.

Time in hours	Carbohydrates A	Carbohydrates F	Nitrogen GA	Nitrogen AS	pH	C:N	g% Biomass	g% Protein	Yield
0	4.25	1.42	.066	.071	5.15	11.5			
3.6	4.6	2.30	.066	.060	5.2	13			
* 17.5	3.6	2.40	.049	.026	5.1	15.9			
42.1	3.2	2.12	.029	.032	4.9	16	1.07	29.76	0.95

* Aeration increased to 600 ml/min N.T.P. sinter starting to clog.

table 3

Control

Organism: AD_1.

Temperature 30°C; Aeration 490 ml/min N.T.P. Pitch volume 970 ml.

Time in hours	Carbohydrates A	Carbohydrates F	Nitrogen	pH	g% Biomass	g% Protein	Yield
0	4.25	1.42	0.0126	5.15			
3.6	4.8	2.4	0.0	5.0			
17.5	4.2	2.96	0.0	5.2			
42.1	4.1	2.98	0.0	5.3	0.75	16.0	

table 4

Organism: AD_2

Temperature 30°C; Aeration 490 ml/min N.T.P. Pitch volume 950 ml.

Time in hours	Carbohydrates A	F	Nitrogen GA	AS	pH	C:N	g% Biomass	g% Protein	Yield
0	4.25	1.42	.065	.071	5.15	11.5			
3.6	4.5	1.46	.039	.034	5.2	21			
17.5	2.1	0.92	.033	.017	4.5	12			
42.1	1.5	0.48	.007	.017	4.0	17	1.8	20.9	0.65

table 5.

Control

Organism: AD_2

Temperature 30°C; Aeration 490 ml/min N.T.P. Pitch volume 950 ml.

Time in hours	Carbohydrates A	F	Nitrogen	pH	C:N	g% Biomass	g% Protein	Yield
0	4.25	1.42	0.012	5.15				
3.6	4.6	1.40	0.003	5.0				
17.5	4.0	1.06	0.001	4.9				
42.1	3.2	0.94	0.001	4.9		0.95	8.75	0.9

table 6.

Organism: AD_3

Temperature 30°C; Aeration 490 ml/min N.T.P. Pitch volume 975 ml.

Time in hours	Carbohydrates A	F	Nitrogen GA	AS	pH	C:N	g% Biomass	g% Protein	Yield
0	4.25	1.42	.072	.065	5.15	11.5			
3.46	4.6	1.6	.062	.048	5.15	15.0			
18.04	4.6	2.3	.065	.045	4.8	15.0			
24.04	4.1	2.68	.060	.045	4.7	14.0			
28.04	3.7	2.68	.058	.045	4.6	12.5			
42.44	2.7	2.3	.058	.034	4.6	10.0	0.51	44.64	0.33

table 7.

Control

Organism: AD_3

Temperature 30°C; Aeration 490 ml/min N.T.P. Pitch volume 975 ml.

Time in hours	Carbohydrates A	F	Nitrogen	pH	C:N	g% Biomass	g% Protein	Yield
0	4.25	1.42	0.0126	5.15				
3.46	4.8	1.56	0.0042	5.15				
18.04	4.6	2.24	0.0014	4.8				
24.04	4.4	2.96	0.001	4.7				
28.04	4.1	2.68	0.001	4.65				
42.44	4.1	2.96	0.001	4.7		0.49	18.64	0.7

table 8.

7.5 Chromatographic Carbohydrate Analyses of carob media before and after fermentation by AD_1, AD_2 and AD_3.

	Initial Carbohydrate concentration of carob media.	Final Carbohydrate concentration of carob media after fermentation by AD_1, AD_2 and AD_3.		
		AD_1	AD_2	AD_3
	g%	g%	g%	g%
Glucose	0.79	1.246	0.27	0.95
Fructose	0.88	1.29	0.25	1.179
Maltose	0.49	0.419	0.30	0.476
Sucrose	2.30	0.269	0.30	0.299

table 9

The percentage recovery of the standards glucose, fructose, maltose and sucrose from the chromatogram was 86.73 g%, 91.84 g%, 86.50 g% and 76.53 g% respectively. Therefore the appropriate losses were taken into account in the determination of the individual carbohydrates in the carob media before and after fermentation.

7.6 Selection of a Fungus for further analyses

The criteria used for the selection of a fungus for further analyses are as follows:

(i) g% Total Nitrogen utilized

(ii) g% Biomass produced

(iii) g% Protein of the fungal mycelium

(iv) Yield

The selection was made as follows:

Consider g% Carbohydrate utilized; it is an advantage if a smaller proportion is used per gramme of mycelium produced; which is the yield, therefore the fungus with the highest yield was

awarded a score of 1, the next fungus with the second highest yield was awarded a score of 2, and so on. The same scoring system was used for g% protein and g% biomass. The nitrogen utilized was not considered in the same way, because if one assumed that the fungus which utilized the most nitrogen would have the greatest g% protein content we would be incorrect. In fact in the three cases in question AD_1, AD_2 and AD_3 the situation is the reverse, the fungus with the highest g% protein utilized the least nitrogen; therefore the system of scoring was reversed, that is the fungus with the lowest nitrogen utilization was awarded a score of 1 and so on.

	g% Protein	g% Biomass	Yield	g% Nitrogen Utilized	Total Score
AD_1	2	2	1	2	7
AD_2	3	1	2	3	9
AD_3	1	3	3	1	8

table 10

Therefore the fungus with the lowest score was selected for further investigation. As can be observed from the above table 10; the fungus selected was AD_1.

Discussion and Conclusions

The resultant growth of AD_1, AD_2 and AD_3 in Experiment 2sc was similar to that obtained in Experiment 1sc, that is AD_1 and AD_2 were compact rice shaped pellets measuring 2 - 4 mm in length; AD_3 was spherical pellets 2 - 3 mm in diameter.

The protein contents of AD_1, AD_2 and AD_3 in the control experiments 2sc (tables 4, 6 and 8) were 16.0 g%, 8.75 g% and 18.64 g% respectively. The protein contents of AD_1, AD_2 and AD_3 in the actual experiments 2sc (tables 3, 5 and 7) in which glutamic acid and ammonium sulphate was added to the carob media was 29.76 g%, 20.9 g% and 44.64 g% respectively. It was obvious therefore that the addition of one or both of these nitrogenous compounds to the carob media was necessary for protein production.

There was no significant difference between the initial and final pH of the carob media supplemented experiments 2sc, or between the final pH of the control, and the final pH of the carob media supplemented experiments 2sc (tables 3, 4, 5, 6, 7, 8). Since the pH in the control experiments did not change drastically this suggested that no metabolites such as organic acids, which would bring about a change in pH, were excreted into the carob media by the fungi. Therefore the small pH changes encountered were most probably due to the reduction of the carbohydrate and nitrogen concentrations. Whether this also applied to the carob media supplemented experiments is a matter of conjecture, since the glutamic acid and monosodium glutamate present in the media would act as a buffer system. However as the glutamic acid and monosodium glutamate was utilized by the fungi the capacity of the buffer system would decrease, and if such metabolites had been excreted into the media by the fungi that would bring about a change

in pH; the pH change would have been a sharp change when the capacity of the buffer system was exceeded. Since no such changes of pH were recorded it is reasonable to assume that no metabolites such as organic acids were excreted into the supplemented carob media by AD_1, AD_2 and AD_3.

The glutamic acid nitrogen and ammonium sulphate nitrogen in the case of AD_1 and AD_2 in Experiment 2sc was utilized in equal quantities (tables 3 and 5) $\pm$ 0.002 g%, and $\pm$ 0.004 g% respectively. This suggested that both nitrogenous compounds were of equal importance in the production of protein in AD_1 and AD_2. In the case of AD_3 a preference for ammonium sulphate nitrogen was found, approximately twice as much ammonium sulphate nitrogen was utilized by AD_3 than glutamic acid nitrogen, (Experiment 2sc, table 7.) whether or not the glutamic acid in this case was superfluous is difficult to say.

The initial C:N ratios of the supplemented carob media of AD_1, AD_2 and AD_3 (Experiment 2sc) was 11.5 (Experiment 1sc, table 2) It was thought that this C:N ratio would have been a close approximation to the true optimum C:N ratio. If this C:N ratio of 11.5 was a close approximation to the true optimum C:N ratio, it was expected that little change if any, of the C:N ratio would occur during the fermentation, since the carbohydrates and nitrogen would have been utilized at the correct differential rate, thus the C:N ratio staying fairly constant throughout the fermentation. However this was not so, in all cases the C:N ratio changed significantly (Experiment 2sc, tables 3, 5 and 7). In the case of AD_1 it was found that at this C:N ratio of 11.5 the g% biomass produced was greater than that obtained in the first submerged culture experiments (Experiments 1sc.) when the C:N ratio was

between 12 - 13, in both cases the g% protein was approximately the same. In contrast to this the g% protein of AD_2 had been reduced from 30 g% obtained in the first submerged culture experiment (Experiment 1sc.) to 20.9 g% in Experiment 2sc. (table 5.), although the g% biomass had been increased from 1.0 g% in Experiment 1sc. to 1.8 g% in Experiment 2sc. the total protein produced in each case was the same. It did suggest that there is possibly an optimum C:N ratio for biomass production, but not for protein production; the total protein always remaining the same in AD_2 within certain limits. In the case of AD_3 the protein content in Experiment 2sc. (table 7.) was unchanged compared with that obtained in Experiment 1sc. However the g% biomass was reduced in this experiment (Experiment 2sc, table 7.) compared with that obtained in Experiment 1sc. It appeared therefore that the stabilized C:N ratio of 13 obtained in experiment 1sc. was probably closer to the true optimum C:N ratio than 11.5 used in Experiment 2sc.

Although we have considered that the carbohydrates and nitrogen will be utilized by the fungi at a differential rate, which will be an optimum in each case when the carbohydrates and nitrogen are in the correct ratio (C:N), it does not necessarily follow that the C:N ratio will remain constant throughout the fermentation, since the optimum differential uptake of carbohydrate and nitrogen by the fungi may not only depend on the C:N ratio, but also on concentration. That is to say that the C:N ratio of 11.5 may well have been the optimum C:N ratio at the initial carbohydrate and nitrogen concentrations used in Experiment 2sc, but as these concentrations decreased the carbohydrate and nitrogen was not utilized by the fungi at the correct optimum differential rate to

maintain a C:N ratio of 11.5, therefore biomass and protein production would not be at a maximum.

Although in the case of AD_1 and AD_2 the biomass production had been increased in Experiment 2sc. (table 3 , 7.) in the supplemented carob media, one of two, or a combination of the two following possibilities exist. Firstly the large spore inoculum used provided a large number of initial growing points, therefore a slighter greater biomass being produced in a shorter duration. Secondly the initial C:N ratio including the concentration of the carbohydrates and nitrogen was correct for initial optimum differential uptake by the fungi, therefore the optimum differential uptake rate of these nutrients by the fungi decreased at a slower rate in Experiment 2sc, compared with that in Experiment 1sc. where the C:N ratio or the concentration of carbohydrates and nitrogen or both were not optimum. In the case of AD_3 where the g% biomass had been reduced in Experiment 2sc. (table 7.) compared with that in Experiment 1sc. the reverse may have occurred.

The protein contents of AD_1 and AD_3 grown in the supplemented carob media in Experiment 1sc and Experiment 2sc. (table 3 - 7.) were similar within the limits of experimental error. It would seem that the protein content of these two fungi were determined physiologically and providing sufficient nitrogen was present the maximum amount of protein was produced within its physiological limits. However in the case of AD_2 in the supplemented carob media, (table 5.) the protein content obtained in Experiment 2sc. at a C:N ratio of 11.5 was approximately 20 g%, that obtained in Experiment 1sc. at a C:N ratio of 12 - 13 was approximately 30 g%, but the total protein produced in each case was approximately the same, the choice seems to be " quality or quantity ". It is

possible that the correct C:N ratio was 11.5, but the concentration of the carbohydrates and nitrogen was not optimum for protein synthesis.

It is difficult to say without further investigation whether or not all if any of the glutamic acid was used in the synthesis of proteins. It is feasible that part, if not all, of the glutamic acid could have been utilized as an energy source, therefore less carbohydrates would have been utilized; if this was so it would account for the relatively high yield values obtained in Experiment 2sc. for AD_1 and AD_2 (tables 3, 5 and 7) in the supplemented carob media.

From the results of the chromatography (table 9) all the carbohydrates identified, glucose, sucrose, fructose and maltose (ceretose and primverose) were utilized to varying degrees. In the case of AD_1, Experiment 2sc. the estimations of the individual carbohydrates at the end of the fermentation showed that the glucose concentration had increased, compared with its initial concentration, and so had the fructose concentration, but the sucrose concentration had been greatly reduced (table 9). This therefore suggested that sucrose was first split extracellularly, most likely by invertase excreted into the supplemented carob media by AD_1 before direct assimilation of the resulting glucose and fructose.

In the case of AD_2, Experiment 2sc. the situation presented by AD_1 is not so well defined; however the glucose and fructose concentrations were approximately equal to that of sucrose (table 9), which suggested that a similar situation to that found with AD_1 was also applicable to AD_2. The results of AD_3 were directly similar to those of AD_1.

The Fehlings estimation for reducing carbohydrates taken throughout the fermentation showed an increase in reducing carbohydrates in all cases. It therefore appears that the enzyme invertase is an extracellular enzyme, being excreted into the media by the fungi to bring about the enzymic hydrolysis of sucrose, the products of which were then assimilated by the fungi.

Since there was also present in the carob media glucose and fructose before sucrose was enzymically hydrolised, it seems logical metabolically that the free glucose and fructose would be utilized first by the fungi before sucrose was enzymically hydrolized.

Section 8

Growth of AD_1 in carob media supplemented with (i) Ammonium sulphate, (ii) Glutamic acid.

Experiment 3sc. - Submerged Culture.

Experiment 3sc

Growth of AD_1 in carob media supplemented with (i) Ammonium sulphate, (ii) Glutamic acid.

8.1 Experimental design

In Experiment 2sc. (table 3) it was found that AD_1 utilized Glutamic acid nitrogen and Ammonium sulphate nitrogen in equal proportions. This indicated that glutamic acid and ammonium sulphate may be of equal importance in the production of biomass and protein in AD_1. To verify or disprove this the following experiment was carried out, that is AD_1 was grown in (i) carob media supplemented with glutamic acid, (ii) carob media supplemented with ammonium sulphate.

Materials and Methods

The experiments were carried out in 1.01 tower fermenters as before, using the same type of media except that this time one media was supplemented with ammonium sulphate and the second with glutamic acid. The C:N ratio was between 13 and 14.

Carbohydrate estimations, nitrogen determinations and protein estimations were carried out as in the previous experiments.

Results

8.2 AD_1.

Carob media supplemented with Ammonium sulphate.

Temperature 30°C; Aeration 600 ml/min N.T.P. Initial C:N ratio 13.

Time in hours	g% Carbohydrates	g% Nitrogen	pH	g% Biomass	g% Protein	Yield
0	4.8	0.146	5.1			
43	2.6	0.007	4.0	0.32	32.6	0.15

table 16

AD_1.

Carob media supplemented with Glutamic acid.

Temperature 30°C; Aeration 600 ml/min N.T.P. Initial C:N ratio 14.

Time in hours	g% Carbohydrates	g% Nitrogen	pH	g% Biomass	g% Protein	Yield
0	4.4	0.120	5.1			
43	2.9	0.058	5.3	0.48	34.0	0.3

table 17

Discussions and Conclusions

As can be observed from table 16 the protein content produced by AD_1 in the carob media supplemented with ammonium sulphate was 32.6 g% protein; compared with 34.0 g% protein obtained when grown in carob media supplemented with glutamic acid (table 17). This difference of 1.4 g% protein is not significant; however when compared with that obtained in Experiment 1sc. and Experiment 2sc. (table 3), when AD_1 was grown on carob media containing both these nitrogenous compounds the protein content was 30 g%, a difference of 2 - 4 g% protein, which may be significant. If we consider the growth of AD_1 in Experiment 3 sc on the carob media supplemented with (i) ammonium sulphate, (ii) glutamic acid, the g% biomass produced was 0.32 g% and 0.48 g% respectively and the yield was 0.15 and 0.3 respectively. However in Experiment 1sc. and Experiment 2sc. (table 3) when AD_1 was grown on carob media containing both these nitrogenous compounds, the g% biomass was 0.7 g% and 1.0 g% respectively and the yield was 0.34 and 0.9 respectively.

Therefore what we may sacrifice for a slightly higher protein content, would result in having a reduction of total protein. It was obvious therefore that ammonium sulphate and glutamic acid were both necessary for biomass production, even if not for protein synthesis.

It may be argued that in Experiment 3 sc. the carbohydrate and nitrogen concentrations were not identical, a difference of 0.4 g% and 0.026 g% respectively, therefore a difference of 1.0 in the C:N ratio. Such small differences are negligable, since a difference in C:N ratio of 2 - 3 is required before any measurable difference in terms of growth and protein content will occur in

submerged culture of fungi. This also applies to any effect due to the small differences in concentration of the nutrients in this case.

Section 9

Production and Preliminary Protein Analysis of AD_1 for Preliminary Feeding Trials.

Production and preliminary protein analysis of AD_1 for preliminary feeding trials.

9.1 Experimental design

The production of fungal biomass of AD_1 was carried out in four 50.01 tower fermenters of similar design to the 1.01 tower fermenters used in the secondary screening, Experiments 1sc., 2sc. and 3sc. A sample of the fungal biomass of AD_1 was taken for dry weight determinations, protein estimations and nucleic acid estimations. The nucleic acid estimation was expressed in terms of total nitrogen. A sample was also taken for amino acid analysis. These estimations were carried out in order to obtain a more realistic value for the protein content of AD_1, also to ascertain the type of amino acids present in a pepsin extract of AD_1. A pepsin hydrolysate was made since the amino acid analysis would reflect more realistically the biological availability of the protein content in AD_1 compared with other more severe types of extraction methods. The remainder of the mycelia of AD_1 was mixed 50/50 with normal laboratory mouse diet to be used in the preliminary feeding trials.

Materials and Methods

The carob media was prepared as in Experiments 1sc, 2sc. and 3sc., and supplemented with glutamic acid and ammonium sulphate in equal quantities in terms of nitrogen content. The C:N ratio of the media was between 12 - 14. The fermentations were allowed to run for 42 hours. Samples of the mycelia of AD_1 were then taken for protein analysis. No parameters during the course of the fermentation were measured, since the prime object was to produce mycelia of AD_1 for protein analysis and preliminary feeding trials. The methods used for carbohydrate, nitrogen and protein estimations were those that have been used throughout this investigation (Chemical Analyses Section 3.).

50.0 litre tower fermenter

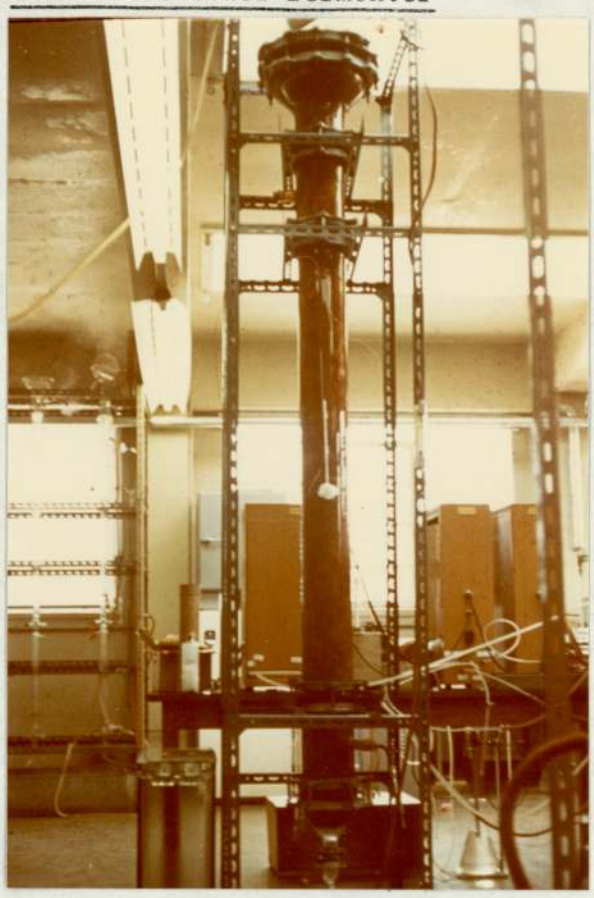


Plate 3

9.2 Inoculum for 50.01 tower fermenters

The inoculum for the 50.01 tower fermenters was a spore inoculum grown on barley grains at 30°C. Glycerol and asparagine was added to the barley grains to induce sporulation in AD_1. The spores were washed off the barley grains aseptically through a buckner funnel into a flask with sterile water. The wash water contained tween '80' to prevent the spores adhering to each other and forming clumps. The spore suspension was then transferred aseptically to the 50.01 tower fermenters.

9.3 Nucleic acid extraction

The nucleic acid extraction was a modified method of Ceriotti, R. (1955). A finely ground sample of AD_1 mycelia which had been dried for three days at 60 - 70°C, whose water content was known was added to 50 ml of ice cold ethanol and allowed to extract for 1.0 hour at 0°C; the whole being gently agitated by a mechanical stirrer. The ethanol extract was then discarded and the residue extracted 3 times with ethanol - ether (ratio 3:1) at 90°C for 3.0 minutes. The residue was weighed and a sample taken for dry weight determinations. To a second sample of this residue was added 25 ml of ice cold 2% perchloric acid and extracted for 20.0 minutes at 0°C, the extract was discarded and the extraction repeated. The residue - defatted deproteinised mycelia of AD_1 was then extracted twice with 70 ml of 10% perchloric acid at 70°C for 20.0 minutes, to remove the nucleic acids. At the end of each extraction the supernatants were collected after centrifuging. The residue was washed with 10% perchloric acid and added to the combined supernatants containing the nucleic acids. Nitrogen determinations were carried out on 1 ml samples of the nucleic acid extract by the Kjeldahl method.

9.4 Amino acid analysis

The amino acid analysis was carried out using an EEL 194 automatic amino acid analyser. A sample of AD_1 mycelia was mixed in a Waring blender with 80 ml of distilled water for 2.0 minutes, washed out of the Waring blender with another 80 ml of distilled water into a 250 ml beaker, thus the total volume of distilled water was 160 ml. Then 0.10 g% (of 160 ml) of pepsin (Emiliani, E. and Ucha de Davie, I. 1962) was added to 40 ml of distilled water which was then transferred to the 250 ml beaker, the contents of which were gently agitated by a mechanical stirrer. The pH was then adjusted to pH 3 with concentrated hydrochloric acid, since this is the optimum pH for this enzyme. The pepsin hydrolysis was allowed to continue for 24 hours, after which the contents of the 250 ml beaker was filtered. The dry weight of the residue was noted, and the filtrate concentrated by vacuum evaporation to 50 ml in a rotary evaporator. This concentrate was then made up to 100 ml with washings from the evaporating flask of the rotary evaporator, and the pH adjusted to 2.2 for amino acid analysis. A sample of the prepared amino acid extract was then diluted 25 x and 1.0 ml of this was used for the analysis of the acidic and neutral amino acids. A second sample of the original amino acid extract was diluted 50 x and 1.0 ml of this was used for the analysis of the basic amino acids.

The procedure for calculating the amount of amino acids present was as follows. The average base line for each peak was found by taking the mean of the first and final base readings for that particular peak. The true height of the peak was then found by subtracting the average base line value from the maximum recorded peak height. The half peak height was taken and the number of dots counted in the upper half of the peak. This value

was then multiplied by the true height of the peak ($\frac{1}{2}$ base x height). The value obtained for the standard peak was then divided into the value obtained for the unknown; (AD_1) since the standard amino acid concentration was 0.1 μ moles, except for proline which was 0.2 μ moles the amino acid concentrations of the unknown (AD_1) was calculated.

Results

9.5 Kjeldahl protein content of AD_1.

The average protein content of AD_1 from the four 50.01 tower fermenters was 36.5 g%.

9.6 Nucleic acid extraction and nitrogen determinations

Weight of AD_1 taken for extraction = 1.40315 g

Water content of AD_1 = 8.33 g%

Actual dry weight of AD_1 = 1.28627 g taken for (i) ethanol (ii) ethanol-ether extractions.

Weight of AD_1 residue = 1.36055 g

Water content of AD_1 residue = 7.32 g%

Actual dry weight of AD_1 residue = 1.26096 g

$\therefore$ loss of AD_1 in extraction = 0.02531 g

Weight of AD_1 residue taken for further extraction = 0.5561 g

Actual dry weight (7.32 g% water) = 0.5154 g

but this has been partly extracted (defatted)

$\therefore$ Actual weight would have been

$$0.5154 + \frac{0.2531}{1.28627} \times 0.5154 = 0.5255 \text{ g}$$

Final volume of extract = 78.0 ml

Nitrogen content in 1.0 ml = 0.0084 g%

Total nitrogen in 78 ml = $\frac{0.0084 \times 78}{100}$

= 0.006552 g.

g% nitrogen due to nucleic acids in 0.5255 g of AD_1

$$\frac{0.006552}{0.5154} \times 100 = 1.2468 \text{ g\%} \equiv 7.79 \text{ g\% protein}$$

The estimated Kjeldahl protein content of AD_1 was 36.4 g%, but 7.79 g% was due to nucleic acids thus the Kjeldahl protein of AD_1 corrected for nucleic acids is 28.61 g%.

9.7 Amino acid analysis

Pepsin extract.

Weight of AD_1 taken for extraction = 5.4490 g

Water content of AD_1 = 7.7481 g%

Actual dry weight of AD_1 taken for extraction = 5.0268 g

After pepsin hydrolysis dry weight of residue of AD_1 = 3.0526 g

Weight of AD_1 in solution = 1.9742 g

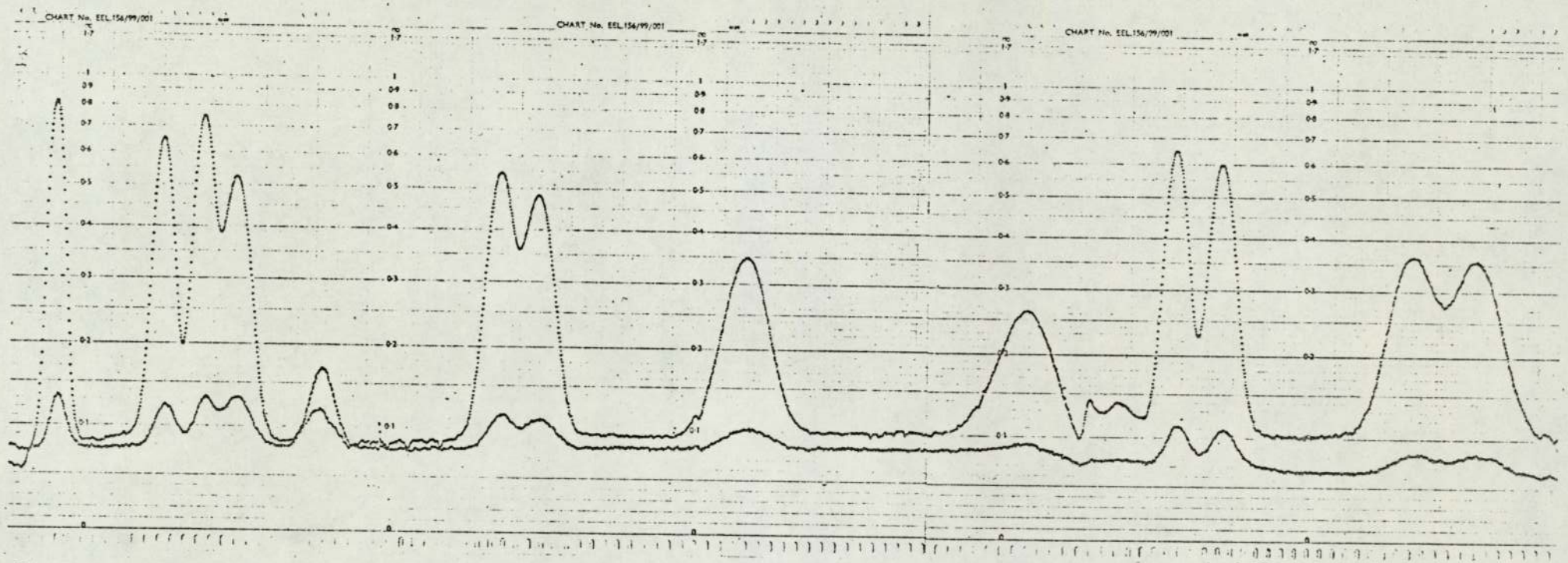
Since this was in 100.0 ml, 1.9742 g% of AD_1 in solution.

9.8 Amino acid concentrations in 1.9742 g of AD_1.

Amino acids	μ Moles	Neutral & Acidic Amino acids	μ Moles
Aspartic	1979.04	Valine	3400.02
Threonine	3047.59	Methionine	624.31
Serine	207.90	Isoleucine	2937.02
Glutamic	15646.68	Leucine	6086.26
Proline	1612.30	Tyrosine	2193.72
Glycine	1648.50	Phenylalanine	3232.35
Alanine	7191.20		

Standard trace of acidic and neutral amino acids.

1 ml sample containing 0.1 moles per amino acid except proline which was 0.2 Moles



Standard amino acids in order of separation (peaks):

Aspartic, Threonine, Serine, Glutamic, Proline, Glycine, Alanine, Cystine, Valine, Methionine, Isoleucine, Leucine, Tyrosine and Phenylalanine.

Standard trace of basic amino acids.

Standard amino acids in order of separation (peaks).

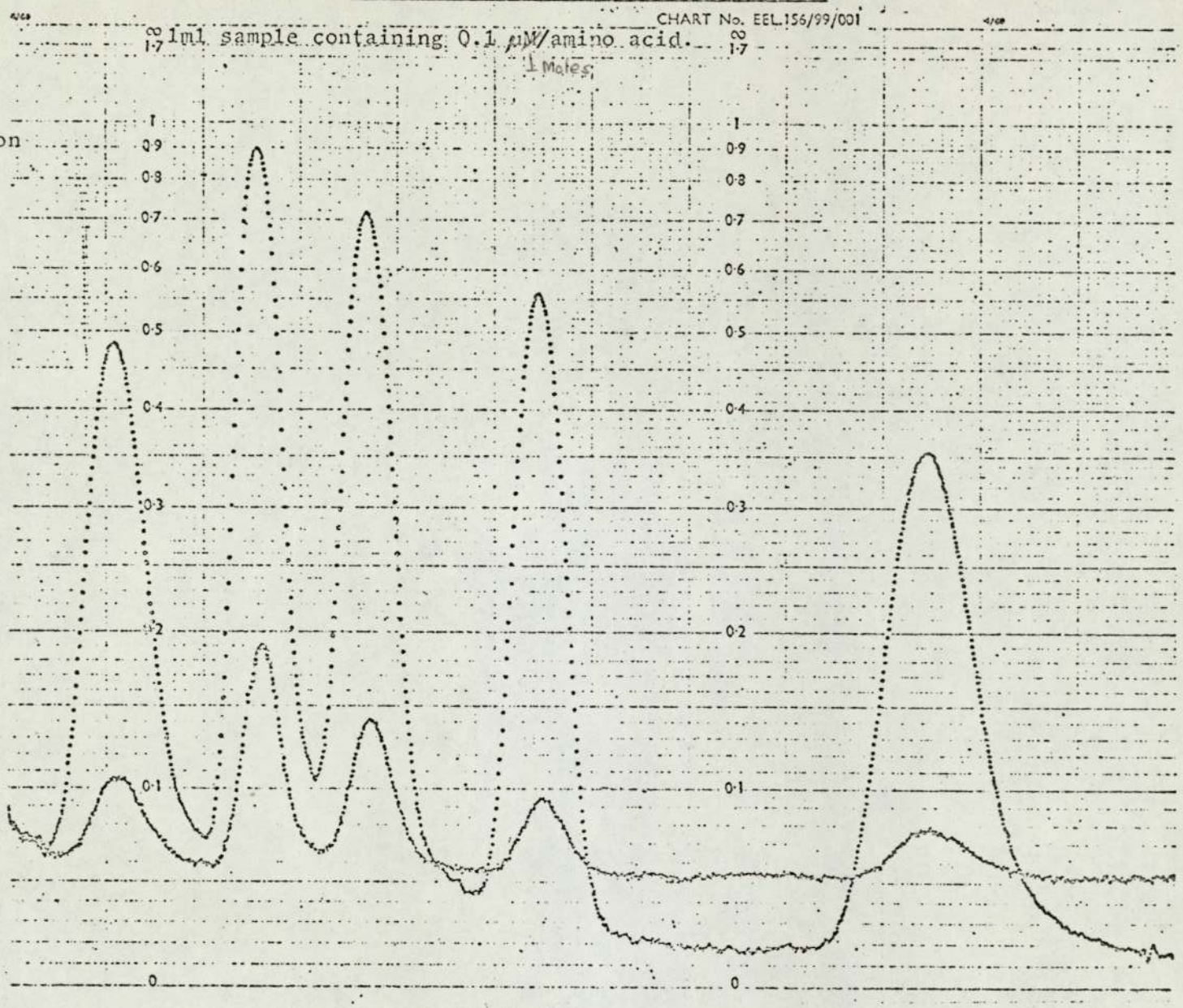
Tryptophan

Lysine

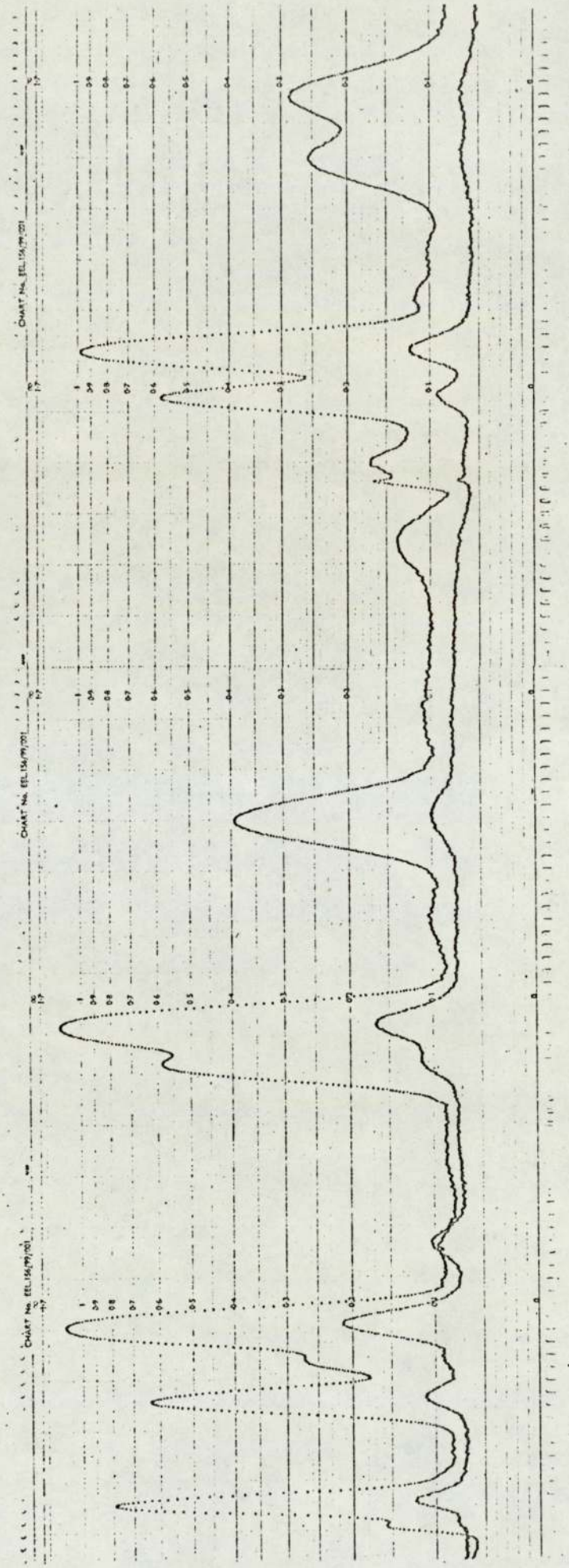
Histidine

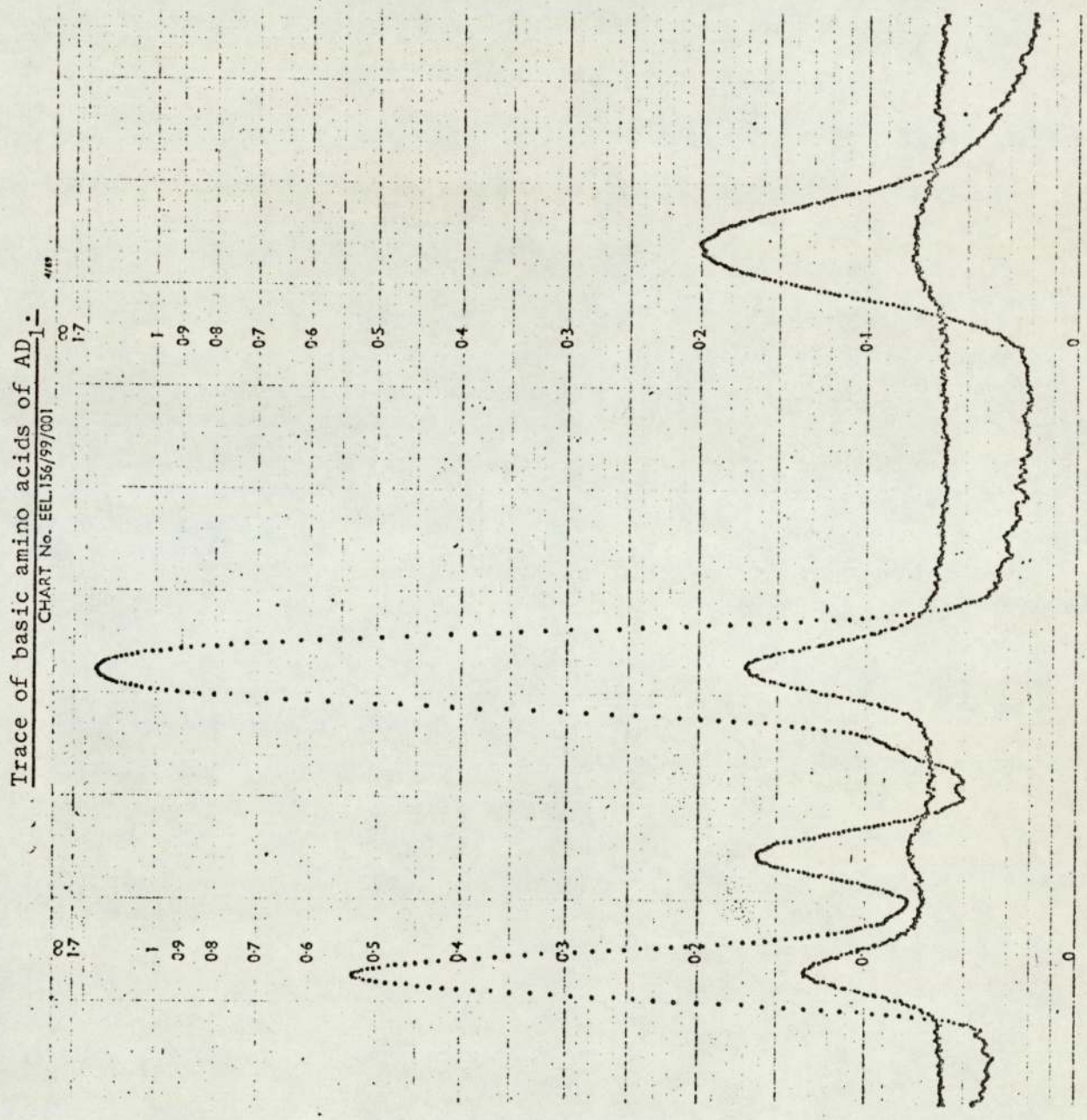
Ammonia

Arginine



Acidic and neutral amino acid spectra of AD_1.




Trace of basic amino acids of AD 1-
CHART No. EEL.156/99/001
∞
1·7
1
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0

Basic

Amino acid.	μ Moles
Lysine	4387.30
Histidine	1116.00
Arginine	5106.90

Sum total of amino acids = 0.004980689 g/ml.

∴ in 100 ml 0.4980689 g

$$\therefore \frac{0.4980689}{1.9742} \times 100 = 25.23 \text{ g\%}$$

∴ 25.23 g% amino acids in 1.974 g of AD_1.

Discussion and Conlusions

The Kjeldahl protein content of AD_1 from the 50.01 tower fermenters was 36.4 g% compared with approximately 32.0 g% in Experiments 1sc. and 2sc. when cultured in the 1.01 tower fermenters. This increase in Kjeldahl protein of AD_1 may be attributed to the increased oxygen transfer rate which would prevail in the 50.01 tower fermenter compared with the 1.01 tower fermenter.

Since the Kjeldahl protein of AD_1 was 36.4 g% and 7.79 g% of this was due to nucleic acid nitrogen, therefore 28.61 g% remaining and of this, 25.23 g% was available as actual amino acids from the pepsin extract, thus a deficit of 3.38 g%. However the cystine and tryptophan content of AD_1 was not measured due to their instability; but it is highly unlikely that they would have constituted 3.38 g% of the amino acids of AD_1 from the pepsin hydrolysate extract. Therefore the greater proportion of the 3.38 g% deficit may have been due to unavailable amino acids in the cell walls of AD_1 and non-protein nitrogen.

Section 10

Preliminary nutritional and toxicological evaluation of AD_1.

Preliminary nutritional and toxicological evaluation of AD_1.

10.1 Introduction

Since the object of this experiment was the preliminary nutritional evaluation of AD_1 protein, the preliminary toxicological evaluation of AD_1, parameters to be measured were chosen which were capable of reflecting either of these effects.

These evaluations of AD_1 were carried out using 21 day old pure bred mice. The reason for using mice, apart from the ease of handling, was that well defined inbred strains were available and the mouse is better known than the rat from a genetical point; they also have similar needs for vitamins and amino acids as rats. (Munck, L. 1968). Mice that had just been weaned (21 days old) were taken for the experiment because studies have shown that neonatal animals generally have a lower capacity to detoxify drugs, (Os'er, B. L. 1968) also any growth inhibitory agents present would have their effects amplified in this period of rapid growth of the mice.

It was suggested by Kosterlitz, H. W. (1944) that the nitrogen content of the liver responds to different dietary proteins, and may therefore be used as a basis for determining the nutritive value of proteins; however Rippon, W. P. (1959) showed that liver nitrogen appeared to be unreliable as a measure of protein quality. It was decided to measure the liver nitrogen content to see if there was any relationship with protein quality; but it was also selected as a parameter to be followed on the grounds of toxicity. If the liver for example is detoxifying amino toxins it is possible that the liver nitrogen may increase, depending on the degree and type of toxicity. The liver dry weight was also recorded since this will be effected not only by toxins but also by

the nutritive value of proteins.

Munchow, H. and Bergner, H. (1967) found that serum urea levels were almost inversely proportional to the biological value of the protein, and the reciprocal value of the serum urea levels were found to be correlated with the biological value of the protein. Since urea is the major end product of protein metabolism which is excreted by the kidneys, the serum urea level therefore also serves as a sensitive indication of renal function. An elevated or decreased serum urea level may result from primary renal disease or from extra-renal conditions which disturb kidney function, such as toxins. Based on these facts the serum urea levels of the mice were measured.

Two serum enzyme levels were also measured, isocitrate dehydrogenase (ICD) and leucine amino peptidase (LAP). ICD is a member of the citric acid cycle and belongs to the main chain of enzymes in intermediary metabolism, the highest activity being found in the liver. ICD is found in both the cytoplasm and mitochondria of the cell, but only the cytoplasmic isoenzyme is found in the serum. The life span of unbound ICD is short, having a half life of approximately 60 minutes; so that slight or transient rises in ICD can often only be detected with difficulty. However the determination of ICD is particularly useful in the diagnosis of liver disease, especially in acute intoxication when the highest activities of ICD are found. Rises in ICD activity are also found in myocardial infarction, pernicious anaemia and in malignant tumors in which an elevation of serum ICD indicates the presence of liver metastases.

Determination of serum leucine amino peptidase (LAP) acitivity, or

rather amino acid arylamidase, because there is a difference in the way it splits typical LAP substrates (leucine amide) and synthetic substrates (L-leucine-α-naphthylamide, L-leucine-p-nitranilide). The fact that true LAP does not play a great part in the splitting of leucine-α-naphthylamide or leucine-p-nitranilide does not alter the clinical significance of the test. Increases in LAP activity in serum may be caused by extra-hepatic blockages of the bile duct through concentration oftumors and by intrahepatic cholestasis of a benign or malignant nature. Damage to liver parenchyma with the corresponding cell damage as in acute hepatitis or cirrhosis of the liver can also cause an increase in the LAP activity in serum.

Serum protein determinations were made based on the fact that if the protein of AD_1 in the experimental diet was sufficient then there should be little difference between the results obtained from the control mice. However if the protein of AD_1 was insufficient in the experimental diet, the serum protein may show transient decreases until body protein has been catabolised to replenish the serum proteins. Serum protein levels can also be affected by toxins, for example, in nephrosis which is characterised partly by low serum protein levels as well as low serum urea levels.

Serum amino acid levels are elevated in animals on high protein diets, and depressed when on low protein diets; however, since the serum analysis already mentioned required 3 - 4 mice to be sacrificed at any one time to obtain sufficient serum for analysis; serum amino acid levels were not measured since this would have doubled the number of mice to be sacrificed, and the handling of such an initial large number of mice was not practicable. This is also the reason for not measuring enzyme activity in the liver of glutamate oxalacetic transaminase, which is increased by proteins with a

high biological value (Werthgen, B. and Munchow, H. 1967).

Although the clinical reasons have been given for the estimations of serum urea, ICD, LAP and protein, liver nitrogen and liver dry weight, in the sense that they would be measuring toxicological effects of AD_1 in the experimental diet, the reverse is also true in the sense that they have been chosen to reflect the nutritive value of the protein in AD_1. That is, after a high protein meal, serum urea, serum total protein and liver nitrogen will transiently be elevated and then fall back to normal. The liver weight and body weight of the animal would also increase, and the serum enzyme levels should remain fairly stable within experimental limits.

10.2 Experimental design

The [illegible] mice used in this experiment were T.O. strain. The control mice and experimental mice were composed of equal numbers of male and female, 16 males and 16 females in each case. In both cases the males and females were maintained in separate cages in groups of four. The mice were 21 days old when received and were allowed a three day period on the normal diet to equilibriate before starting the experiment. The mice were then weighed [illegible] every three days thereafter. The group weights of the mice were then expressed graphically against time in days. The group food and water consumption was measured daily, and this was expressed graphically against time in days. After the group weights had been recorded after the first three days from the start of the experiment; one group of mice from the control diet, and one from the experimental diet were sacrificed by ~~etherization~~ anaesthetization, and every three days thereafter. The sacrificed mice were examined for any gross anatomical changes; the liver was removed, dried and weighed, and Kjeldahl nitrogen estimations carried out on samples of the dried livers. The group liver weight and group liver nitrogen of the mice sacrificed was expressed graphically against time in days.

Before removal of the sacrificed mouse livers, or examination for gross anatomical changes, blood was removed from the hearts by cardiac puncture after an incision had been made in the thoracic cavity, and the blood obtained was pooled. The serum obtained from this pooled blood was then examined for urea, ICD, LAP and total protein levels using the Boehringer Mannhein Test Kits.

Materials and Methods

10.3 Control diet

The control diet was 41.B. in pellet form which contained 15 g%

protein, 2.5 g% oil and 5.5 g% fibre. This is the normal diet used in the maintenance of laboratory mice colonies.

10.4 Experimental diet

The experimental diet was a 50/50 mixture of the normal 41.B. diet and AD_1 mycelia which had been grown in submerged culture. The normal diet pellets were ground to a fine powder in the Waring blender as was the mycelia of AD_1; equal quantities of each were then added to the Waring blender and mixed with water to make a stiff paste. This paste was then extruded through a 10 ml syringe in which the exit aperture had been enlarged to approximately the diameter of the normal diet pellets. The extruded paste was then dried at 60 - 70°C for three days, after which it was broken into pieces of approximately the same length as the normal diet pellets. Since the experimental diet was composed of 50% normal diet and 50% mycelia of AD_1, which have protein contents of 15 g% and 25.23 g% respectively; (see protein analysis of AD_1) the calculated protein content of the experimental diet was 20.10 g%. Since mice eat approximately 20 % of their average body weight per day, 4 g of the normal diet per day was fed to the control mice, and 4 g of the experimental diet per day was fed to the experimental mice. Thus we are comparing high and low protein diet effects on mice.

10.5 Serum analysis

Colorimetric measurements of the serum urea levels were made at a wavelength of 546 nm in matched glass curvettes with a 1.0 cm light path at 37°C. A sample of serum was diluted 10 x with physiological saline; to 0.2 ml of this was added 0.10 ml of 0.2 g% urease in 0.05 M phosphate buffer, pH 6.5. A standard was made containing 0.2 ml of 0.003 g% urea and 0.10 ml of urease; a blank was also made containing 0.10 ml of 0.2 g% urease used in preparation of

the serum sample and standard. The test tubes and their contents were then incubated for 15 minutes at 37°C, after which to each was added successively 5.0 ml of 0.106 M phenol in 0.17 mM sodium nitroprusside, and 5.0 ml 5 mM sodium hypochlorite in 0.125 N sodium hydroxide, and allowed to incubate for a further 30 minutes at 37°C. Optical densities of samples (E sample) and standards (E standard) were read against the blank. The serum urea was then calculated as follows:

$$\frac{\text{E sample}}{\text{E standard}} \times 30 = \text{mg urea per 100 ml serum.}$$

Spectrophotometric measurements were made of isocitrate dehydrogenase at a wavelength of 366 nm in matched glass curvettes with a 1.0 cm light path at 25°C. To 2.5 ml of 0.1 M triethanolamine buffer, pH 7.5 containing 4.6 mM D. L-isocitrate and 52 mM of sodium chloride was added 0.5 ml of serum diluted 10x with physiological saline. This mixture was then incubated at 25°C for 5 minutes, after which 0.1 ml of 9.1 mM of nicotimamide adanine di nucleotide containing 0.12 M of manganese sulphate was added. The optical density was then read at exactly 1, 2 and 3 minutes measured against air. The mean of the optical density differences per minute (Δ E/min) was determined and used in the following calculation:

$$\Delta E_{366nM} \text{ min} \times 1879 = \text{mU/ml serum.}$$

Colorimetric measurements were made of leucine amino peptidase (LAP) (leucine arylamidase) at a wavelength of 405 nm in matched glass curvettes with a 1.0 cm light path at 25°C measured against air. To 3.0 ml of 50 mM phosphate buffer pH 7.2, 0.1 ml of 25 mM leucine-p-nitranilide was added and the whole incubated at 25°C for 5 minutes; after which 0.1 ml of serum which had been diluted 10x with physiological saline was added and the optical density

taken (E_1). Exactly 30 minutes after the first reading a second reading was taken.

$$(E_2 - E_1) \times 108 = \text{mU/ml serum.}$$

Colorimetric measurements of total serum protein were made at a wavelength of 546 nm, in matched glass curvettes with a 1.0 cm light path at 20 - 25°C. To 0.1 ml of serum was added 5.0 ml of 0.1 M NaoH containing 16 mM potassium sodium tartrate, 15 mM potassium iodide and 6 mM copper sulphate. This was allowed to stand at room temperature for 30 minutes and the optical density of the sample (E sample) was measured against the reagent added to the serum sample. A standard was made in the same way using 0.1 ml of a 6 g% protein solution. Since the serum used was haemolysed a sample blank was of 0.1 ml of serum and 5.0 ml of 0.1 M sodium hydroxide containing 16 mM of potassium sodium tartrate was made. The optical density (E blank) of this was measured against distilled water and subtracted from the optical density of the sample.

$$\frac{\text{E sample - E blank}}{\text{E standard}} \times 6 = \text{g protein/100 ml serum.}$$

Towards the end of the experiment preliminary histological examination of randomly selected sections of liver and kidneys were made. The histological fixative used was Bouin's and the stain used was Ehrlich's haemtoxylin. The methods used for fixing and staining were the general standard procedures, no deviation from these were made.

Results

In the early stages of the experiment 23 of the mice maintained on the experimental diet died within seven days, that is, 71.8% of the total colony of 32. Thus interpretation of the results obtained such as body weight and serum analysis were impossible. It appeared therefore that the mycelia in the experimental diet may have been responsible for these terminal toxic effects.

10.6 Results of post-mortem of mice maintained on the experimental diet which were sacrificed, and those on which the toxic effects were terminal, compared to controls.

(i) No visible external damage.

(ii) Underside of skin and capillaries greenish yellow in colour.

(iii) Gall bladder grossly enlarged.

(iv) Liver slightly darker in colour.

(v) Contents of intestines black.

(vi) Intestinal haemorrhage.

(vii) Spleen greatly reduced in size.

(viii) Serum samples contained excessive haemolysis.

(ix) Contents of stomach, intestines and stools contained intact mycelia (AD_1).

table 1

10.7 Behaviour of mice maintained on the experimental diet, compared with controls.

(i) Reduced activity approximately eight hours after ingesting the experimental diet.

(ii) Coma, muscular twitching and convulsions.

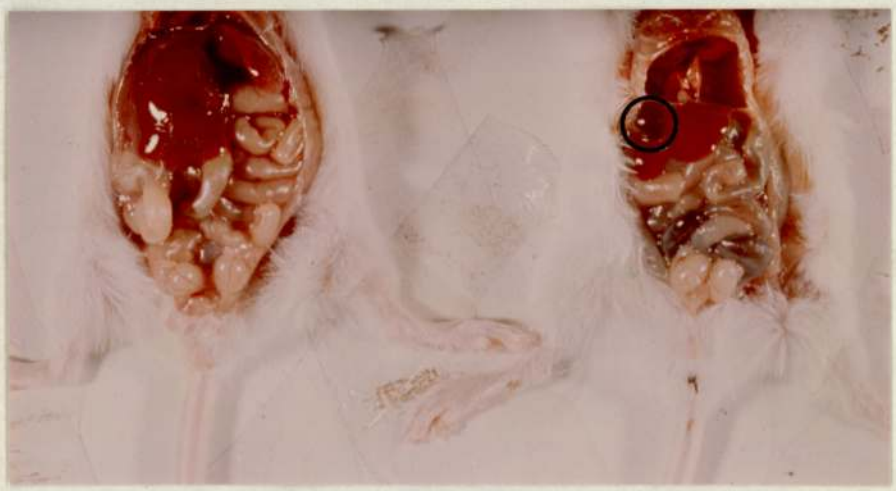
(iii) Gasping type of respiration - usually followed by death.

(iv) Sometimes hind limbs immobile.

(v) Eyes tightly closed or slightly open.

The symptoms (i), (ii) and (iii) were common to all mice on the experimental diet; (iv), (v) and (vi) however were not so common.

table 2



Control Experimental

Plate 1

Liver, gall bladder and intestines of a mouse maintained on the normal diet (Control) for 6 days, compared with the liver, gall bladder and intestines of a mouse maintained on the experimental diet (Experimental) for 6 days.

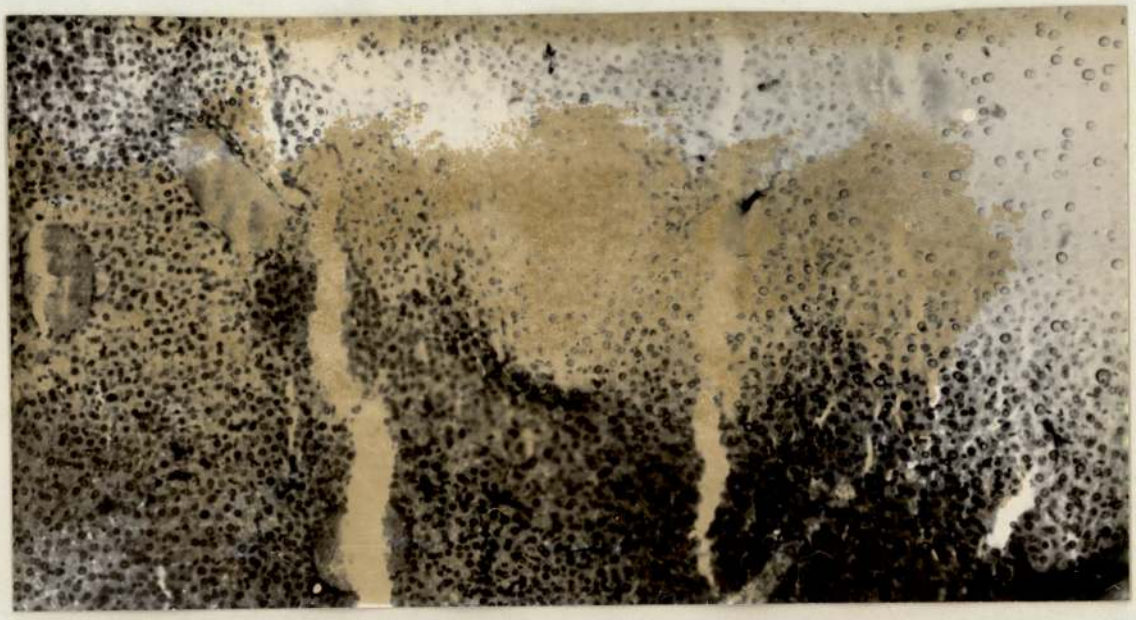


Plate 2.

Section of a liver from a mouse maintained on the experimental diet for six days.

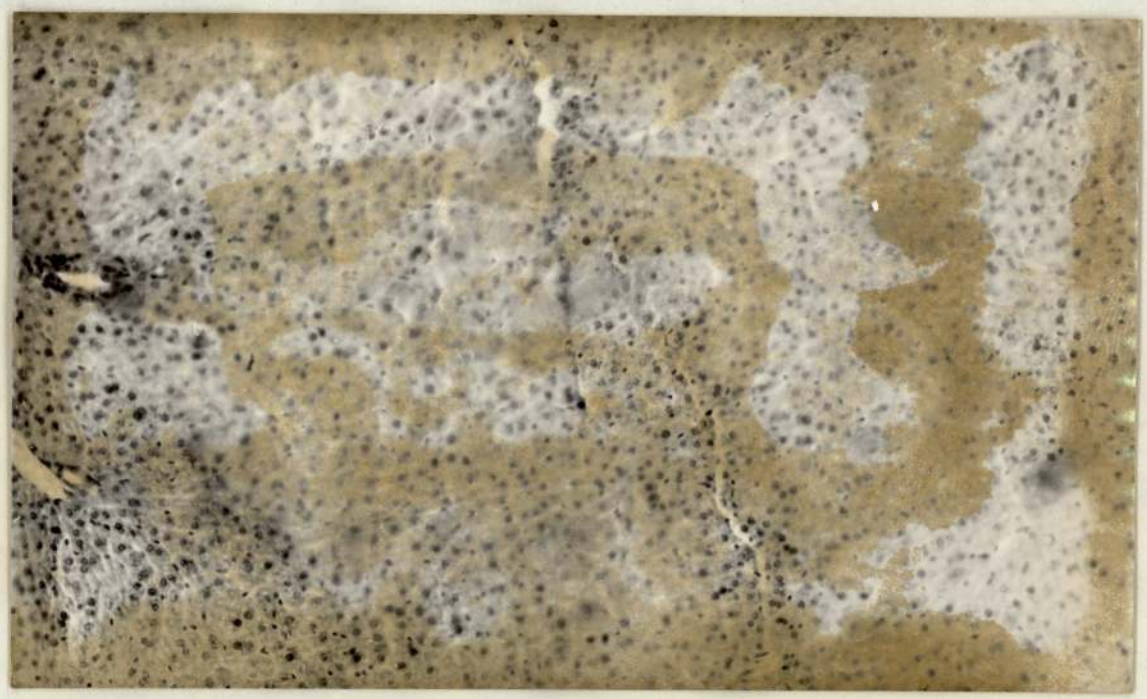


Plate 3.

Section of a liver from a mouse maintained on the control diet for six days.

Plate 4.

Section of a liver from a mouse maintained on the experimental diet for six days.

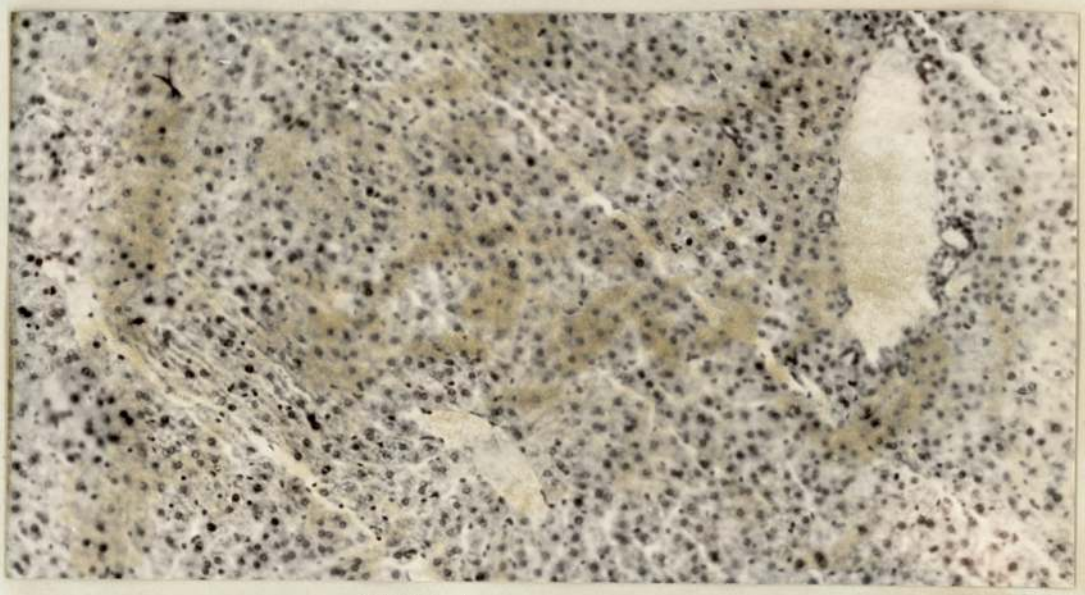


Plate 5.

Section of a liver from a mouse maintained on the control diet for six days.

Experimental

Control

Plate 6.

Spleen of a mouse maintained on the normal diet (Control) for 6 days, compared with the spleen of a mouse maintained on the experimental diet (Experimental) for 6 days.

Discussion and Conclusions

The underside of the skin and capillaries of many of the terminal and sacrificed mice that had been maintained on the experimental diet were greenish yellow in colour, which is an indication of excessive bile pigment, bilirubin in the blood (hyperbilirubinemia) which diffuses out from the capillaries and stains the surrounding tissues; this discolouration is called jaundice. Usually in such circumstances the discolouration is not so intense, but in this case it appeared that the blood may have been excessively overloaded with bilirubin. Jaundice may be caused by the production of bilirubin from the breakdown of erythrocytes in excess of the amount with which the excretory power of the liver can cope, or it may result from the failure of a damaged liver to excrete the bilirubin produced in the normal amounts.

The gall bladder's of the mice maintained on the experimental diet were greatly distended compared to the controls (plate 1.); this may have been caused by a blockage of the common bile duct. However, since the contents of the intestines were black due to excess urobilinogen produced by the reduction of bilirubin (plate 1.) by bacteria in the intestines, also the stools were black due to the high content of urobilin produced by the oxidation of urobilinogen; it is most unlikely therefore that there was any blockage of the hepatic, cystic or common bile ducts resulting in damage to the liver cells. In some of the terminal cases however, the liver was slightly darker in colour (plate 1.) it is possible that in the latter part of the terminal cases liver damage may have occurred resulting in a decrease in liver activity. Histological examination of ten randomly selected livers produced only a few

sections from one liver with possible damage (plates 2, 3, 4, 5.). We may therefore exclude obstructive jaundice as the cause of death.

Since the gall bladders of the mice on the experimental diet were greatly distended and we have excluded obstructive jaundice as a direct cause of death, it appears that the liver was functioning at full capacity and an excessive amount of bilirubin was being produced, a part of which was not taken up by the hepatic parenchyma and was thus therefore retained in the circulation. However the reserve of the liver is so great that it is doubtful whether overproduction of bile pigment ever taxes the excretory power of the liver; but when we consider the size of the spleens which had been greatly reduced in size compared with the controls, (plate 6.) and the fact that the spleen acts as a potential store for erythrocytes, it appeared that the spleen had been almost depleted of this reserve store of erythrocytes. Therefore the evidence suggests that excessive haemolysis had taken place, (this was evident in the serum samples) resulting in the production of bilirubin that was beyond the excretory capacity of the liver.

If we consider the behaviour of the mice on the experimental diet (table 2.) there were three symptoms common to all mice that died; (i) reduced activity approximately eight hours after ingesting the experimental diet; (ii) coma, muscular twitching and convulsions; (iii) gasping type of respiration.

The gasping type of respiration can be brought about by a retention of hydrogen ions which leads to acidosis with a fall in plasma bicarbonate and hence a fall in plasma pH. The muscular twitchings and convulsions can be induced by an imbalance of the blood electrolytes. This behaviour can be accounted for by renal failure due to retention of urea, however the results were

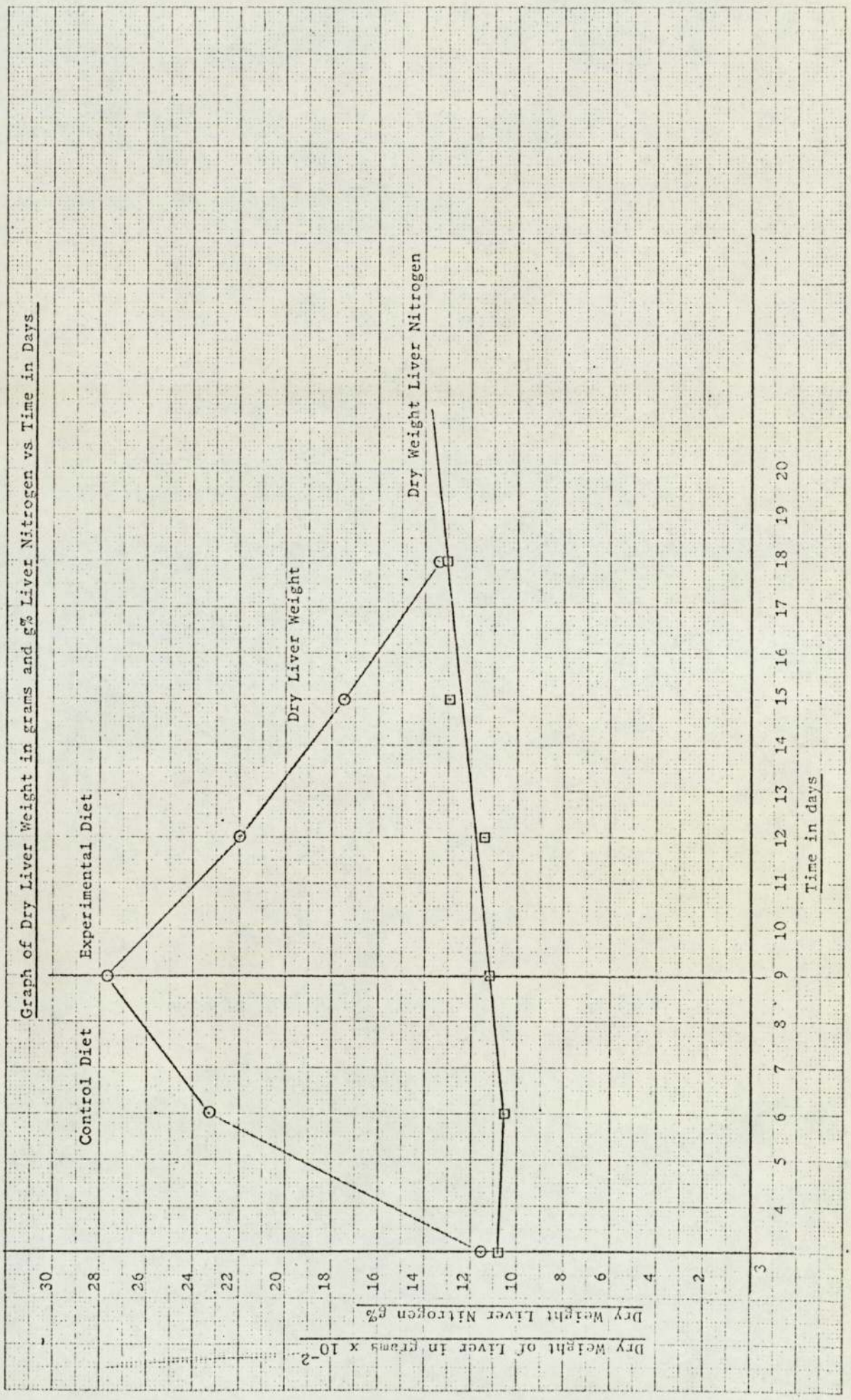
not conclusive because of the lack of data due to the rapid deaths of the mice.

Since the terminal effects were so rapid and severe in the case of the mice maintained on the experimental diet, it was difficult to attribute these results to the experimental diet. It is quite feasible that the cages supplied by the animal house, in which the mice on the experimental diet were kept may have at one time contained animals that were suffering from some disease and the cages were not sterilized correctly. It is also possible that since the cages were made of plastic, chemical sterilization may have been used, and traces of this chemical may have still been present in the cages which proved toxic to the mice.

It was therefore decided to change the cages and transfer the control mice to the experimental diet. If the experimental diet was toxic, the mice which were now 31 days old would be able to exhibit some resistance to the toxin or toxins in the diet.

Section 11

Results of Control mice transferred to the Experimental Diet


Graph of Dry Liver Weight in grams and g% Liver Nitrogen vs Time in Days
Control Diet
Experimental Diet
Dry Liver Weight
Dry Weight Liver Nitrogen
Dry Weight of Liver in grams x 10⁻²
Dry Weight Liver Nitrogen g%
Time in days
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
3
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

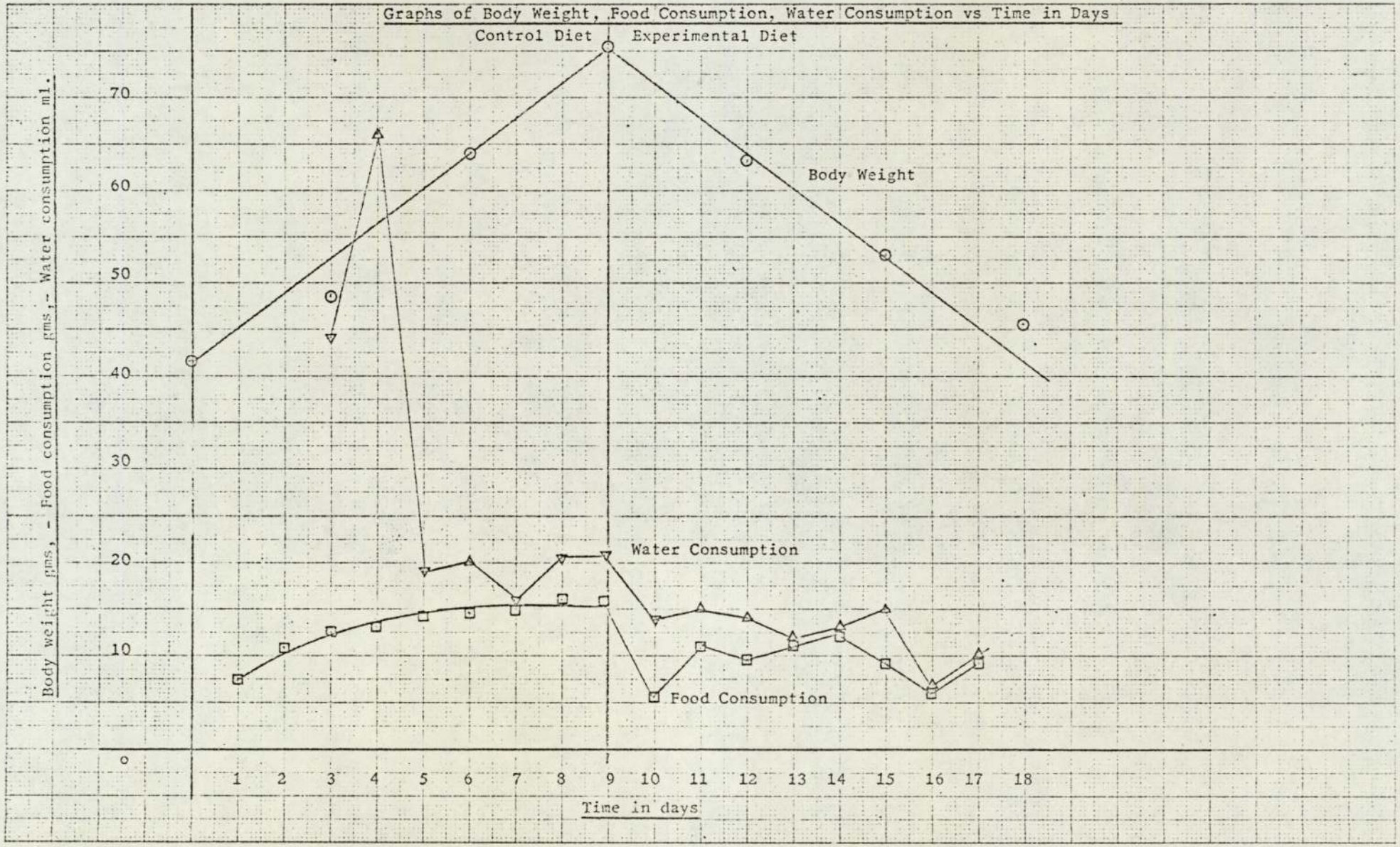
Mice in groups of 4.

Time in days	Group body weight of mice in grams.	Group food consumption of mice in grams.	Group water consumption of mice in ml.
0	41.7	-	-
1	-	7.5	-
2	-	10.8	-
3	48.7	12.9	44
4	-	13.2	66
5	-	14.4	19
6	64.2	14.9	20
7	-	15.0	16
8	-	16.0	21
* 9	75.6	15.6	21
10	-	5.6	14
11	-	11.0	15
12	63.4	9.7	14
13	-	11.1	12
14	-	12.5	13
15	53.10	9.3	15
16	-	6.4	7
17	-	9.5	10
18	45.5	-	-

* Control mice in groups of 4 transferred to experimental diet.

table 4.

As can be observed from the graph the gain in weight of the mice in the first 9 days on the normal diet was four grams per day on the experimental diet. The water and food consumption also decreased when transferred onto the experimental diet.


Graphs of Body Weight, Food Consumption, Water Consumption vs Time in Days
Control Diet
Experimental Diet
Body weight gms, - Food consumption gms,- Water consumption ml.
70
60
50
40
30
20
10
0
Body Weight
Water Consumption
Food Consumption
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Time in days

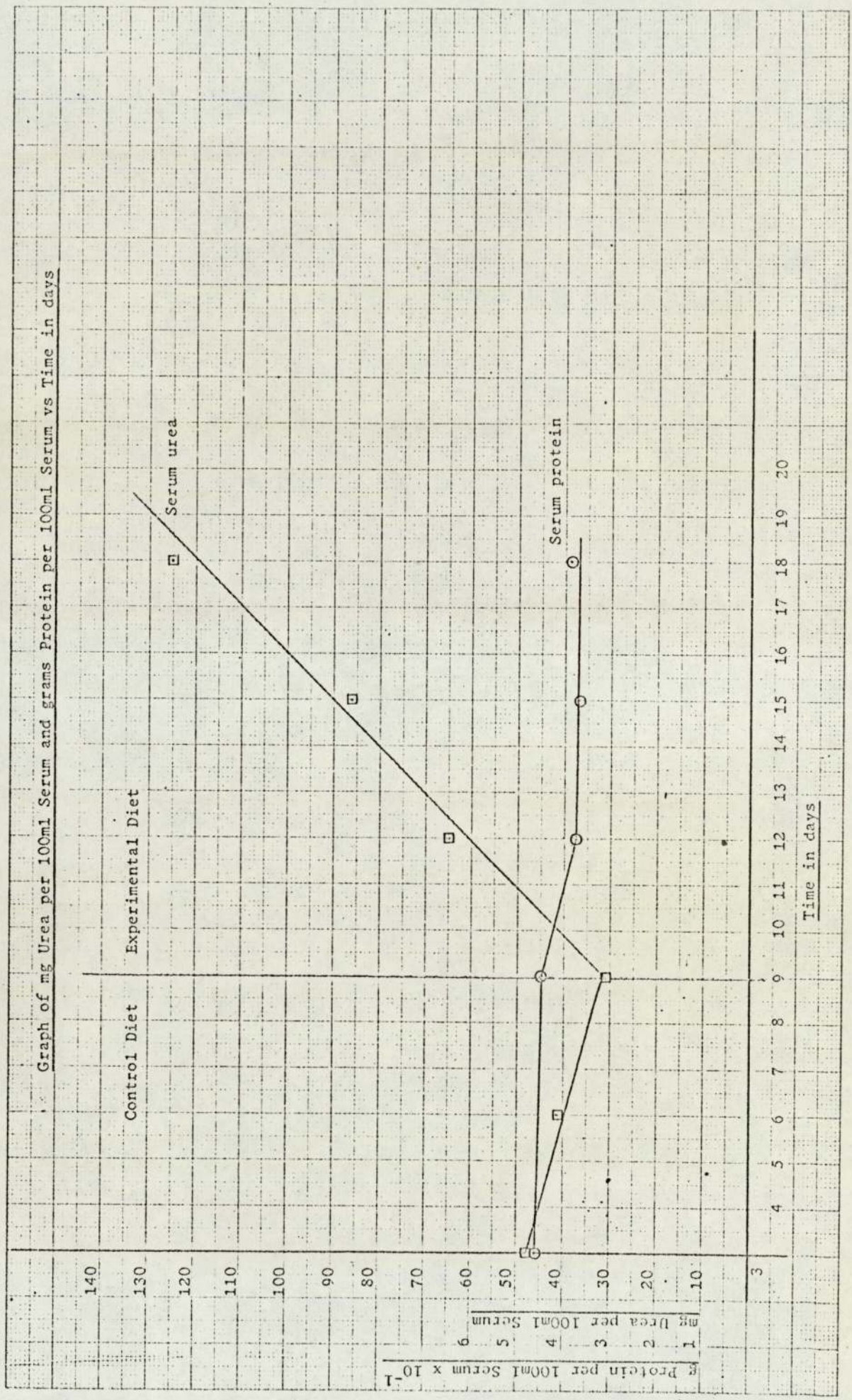
Mice in groups of 4.

Time in days	Number of mice sacrificed	Average mg urea per 100 ml serum	Average grams protein per 100 ml serum
3	4	47.78	4.60
6	4	40.80	-
9 *	3	31.20	4.47
12	3	64.71	3.97
15	3	86.40	3.70
18	2	125.10	3.94

* Control mice in groups of 4 transferred to experimental diet.

table 5.

The serum urea level increased and the serum protein level decreased after the mice were transferred onto the experimental diet.


Graph of mg Urea per 100ml Serum and grams Protein per 100ml Serum vs Time in days
Control Diet
Experimental Diet
Serum urea
Serum protein
mg Urea per 100ml Serum
g Protein per 100ml Serum x 10⁻¹
Time in days

Results of LAP and ICD activity in the serum of sacrificed mice.

Time in days	Number of mice sacrificed.	LAP mU/ml Leucine Arylamidase per group.	ICD mU/ml Isocitrate dehydrogenase per group.
3	4	0.0	61.98
6	4	54.0	0.0
9	3	0.0	30.06
*			
12	3	0.0	62.00
15	3	0.0	46.98
18	2	0.0	10.00

* Control mice transferred to experimental diet.

table 6.

Normal serum LAP values - 8 -22 mU/ml.

Normal serum ICD values - 0 - 7 mU/ml.

It is difficult to analyse the above results of serum LAP and ICD since even some of the results obtained while the mice were on the normal diet are above the normal value. The discrepancy of these results may be attributed to the fact that the serum used in the analysis was not free from haemolysis as is required in such estimations.

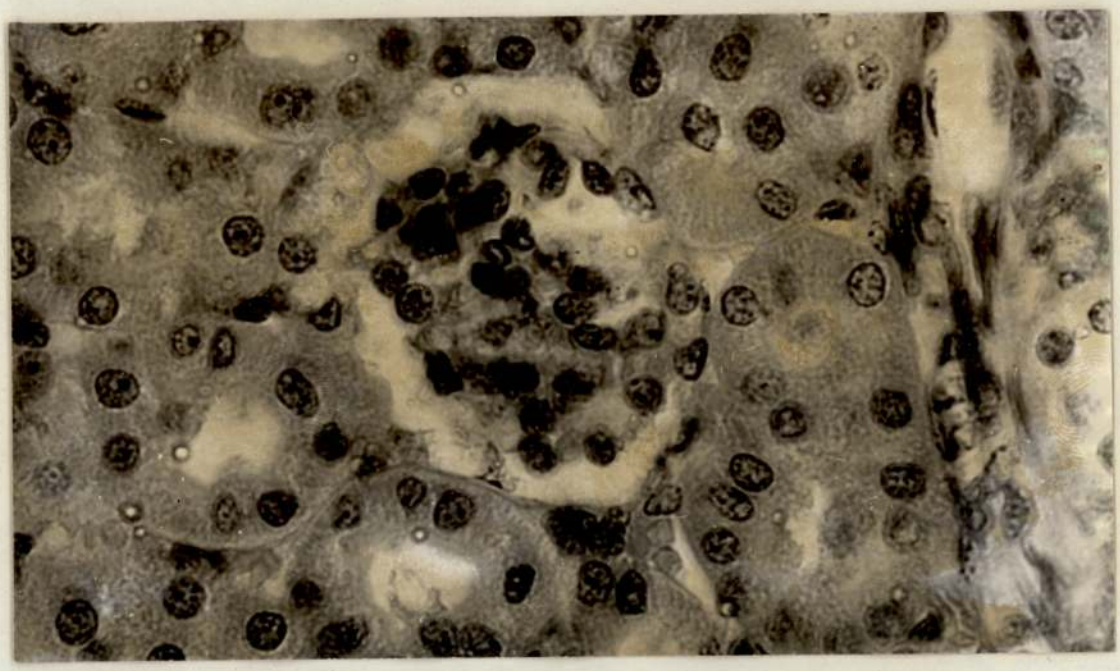


Plate 7.

Section of a kidney from a mouse maintained on the experimental diet for six days.

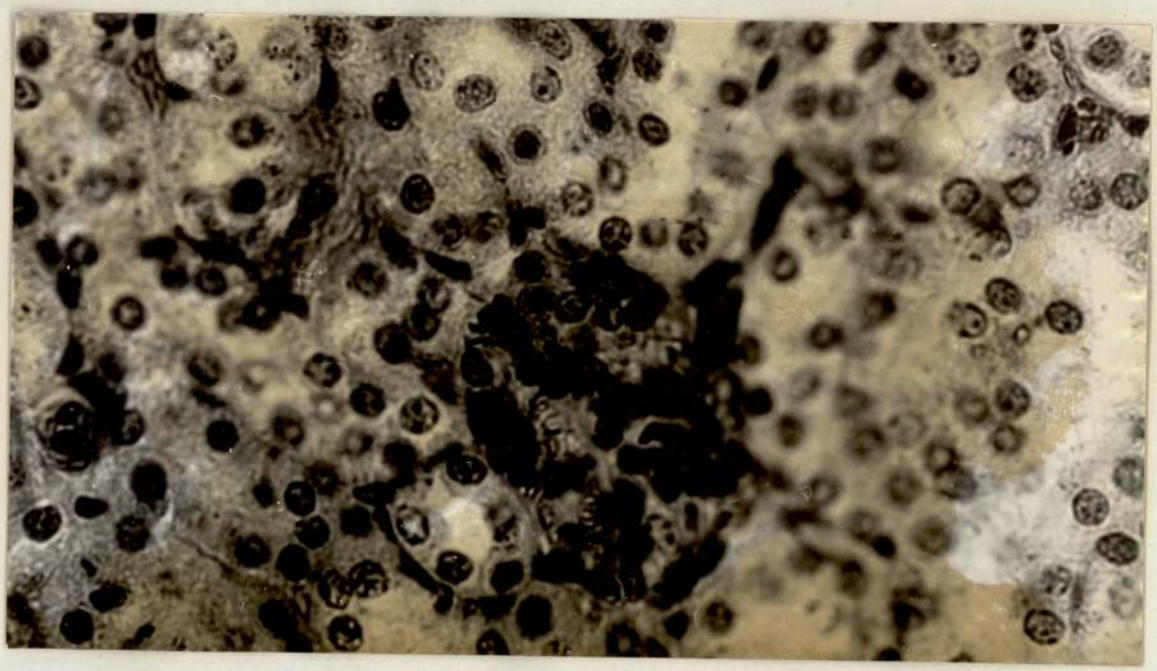


Plate 8.

Section of a kidney from a mouse maintained on the control diet for six days.

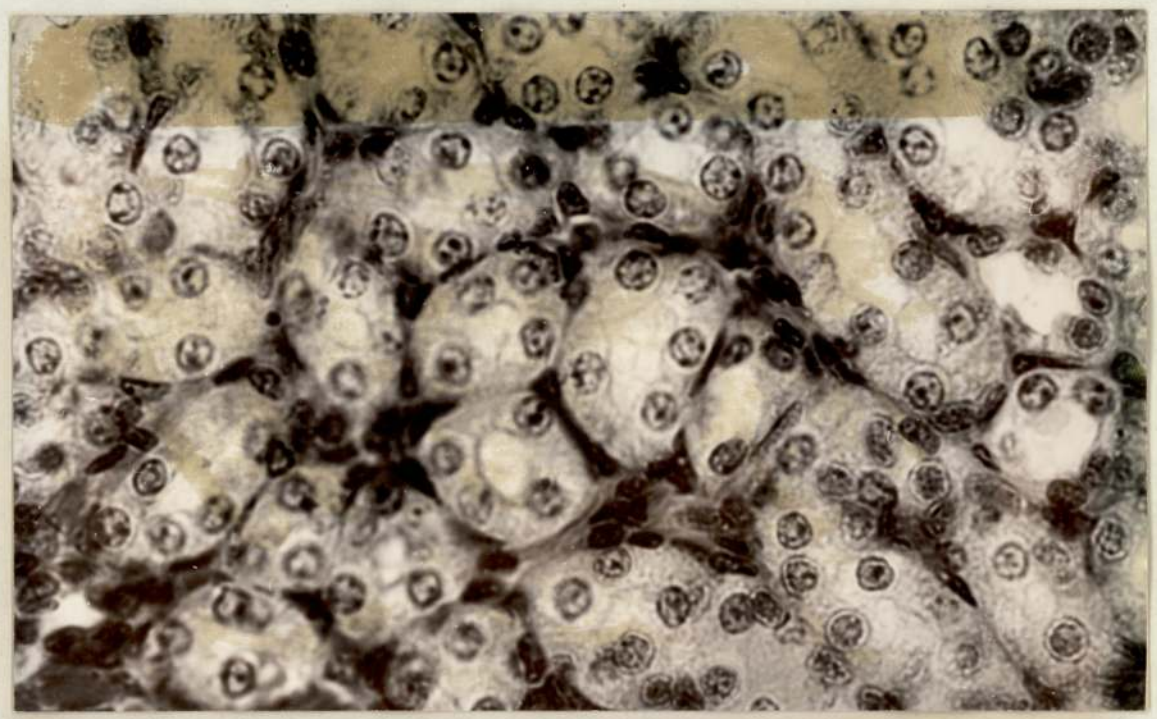


Plate 9.

Section of a kidney from a mouse maintained on the experimental diet for six days.

Plate 10.

Section of a kidney from a mouse maintained on the control diet for six days.

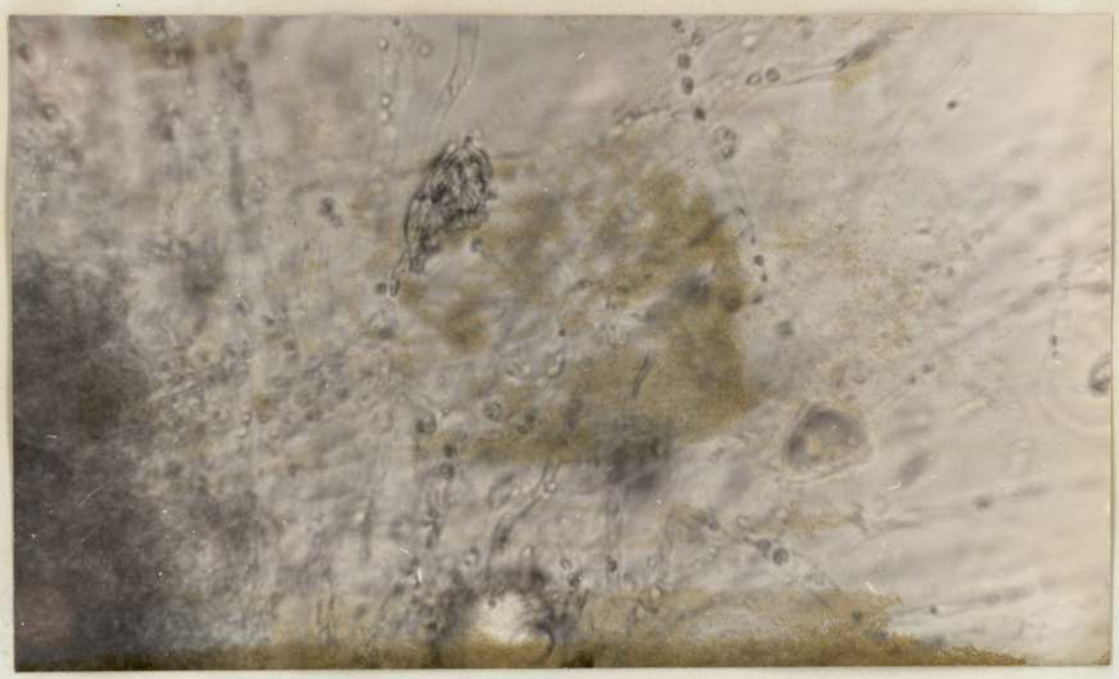


Plate 11.

Stomach contents showing intact mycelia from a mouse maintained on the experimental diet.

Plate 12.

Sample of stool contents showing intact mycelia from a mouse maintained on the experimental diet.

Discussion and Conclusions

Of the 23, 31 day old control mice transferred to the experimental diet, 10 died within 7 days, 31.2% deaths, compared with 71.8% deaths which occurred in the first part of the experiment conducted on 24 day old mice, it is therefore obvious that these deaths in both cases may be attributed to AD_1 in the diet. It appears that the toxin or toxins responsible for these deaths was less effective up to a point, the older the mice became. This suggests that the 31 day old mice were detoxifying in part the toxin or toxins present in the diet. Since the post-mortems of the terminal and sacrificed mice, and their behaviour corresponded with the results obtained in the first part of the experiment carried out on the 24 day old mice; (tables 1 and 2) in which death appeared to have resulted from haemolytic jaundice, the same applies in this case, to some extent. It is therefore possible that the greater survival rate of these older mice may have been due to the fact that they were able to compensate to a greater extent for the loss of erythrocytes by haemolysis, by the now greater capacity of their spleens to store erythrocytes. This capacity being established while on the normal diet.

The increased nitrogen content of the liver (table 3, graph 1) may have been due to the increase in bilirubin content of the liver, due to excessive haemolysis, or it was the result of the liver detoxifying some compound or compounds that were causing haemolysis. The liver weight also decreased when the mice were transferred to the experimental diet (table 3, graph 1), this loss in weight may be attributed to the toxins, or the haemolysis as indeed may be the loss in body weight, which was 4 g per day. This rate of loss in body weight was the same as their initial rate of

gain in body weight when maintained on the normal diet (table 4, graph 2). However when we consider that the food intake had decreased to 44%, (average intake on experimental diet compared to the final value on the normal diet) (table 4, graph 2) obviously this would account in part for the decrease in body and liver weight.

The serum protein level decreased and became stable at a new lower level (table 5, graph 3). This decrease in serum protein is no doubt due to the lack of food intake by the mice rather than some toxic effect, and catabolism of body proteins was maintaining this new low serum protein level. However a low serum protein level is usually an indication of nephrosis, but this is under normal circumstances in which the condition is not complicated by haemolytic jaundice and decreased food intake, besides the final serum urea level was elevated to approximately 3 times its normal value (table 5, graph 3). In nephrosis the reverse occurs.

High urea levels (uremia) is a result of renal failure, this is caused by a considerable decrease in the numbers of functioning nephrons and the balance between urea production and excretion is only maintained by an increased concentration of urea in the blood. Histological examination of 10 randomly selected kidneys did not show any deffinite signs of renal damage (plates 7, 8, 9, 10.).

The behaviour of the mice (table 2,) also corresponded with the clinical features of chronic renal failure. (i) decreased activity, (ii) coma, muscular twitching (iii) gasping form of respiration. The coma and muscular twitching is brought about by an imbalance of blood electrolytes due to the retention of urea, potassium is retained which may lead to cardiac arrest. The gasping type of respiration is due to acidosis. Although the

kidneys are still capable of producing an acid urine they have the reduced ability to produce ammonia, which is therefore not able to combine with hydrogen ions and so total acid excretion is diminished. In addition the tubules may not reabsorb bicarbonate so efficiently, hence a fall in plasma pH. The retention of urea in the blood will cause some haemolysis, however this would not be to the extent to account for the observations on post-mortem examination. The observations made in the post-mortems of intestinal haemorrhage was probably caused by the breakdown of urea to ammonia in the intestine, which is highly irritant.

It is impossible to make any biological evaluation of the protein content of AD_1 in view of these toxic effects. Examination of the stomach and stool contents (plates 11, 12.) revealed that a large portion of the mycelial walls of AD_1 were intact, thus probably limiting the biological availability of AD_1 protein. Since the mycelia of AD_1 was thoroughly washed before being compounded with the normal diet, it is doubtful that the toxic effects observed were due to surface toxins, since these would have most likely been removed in the washings. It is possible that the cellular contents of AD_1 were leached out leaving behind cell walls, thus the majority of AD_1 proteins and toxins becoming available to the mice.

The only conclusion that can be made is that the experimental mice died of haemolytic jaundice and renal failure. The haemolytic jaundice being induced by an haemolysin or haemolysins of AD_1. The renal failure being induced directly by a toxin or toxins of AD_1, or indirectly resulting from detoxification products of the toxin or toxins; or from the detoxification of the haemolysin or haemolysins of AD_1.

It is obvious from the preliminary feeding trials that a modification to the screening of the selected fungi for preliminary nutritional evaluation is required, that is, a rapid way in which to establish whether or not the selected fungi, which were first selected because of their non-toxicity, are still non-toxic after being grown in submerged culture, which is usually an alien environment.

It was therefore decided to feed normal diets containing (i) AD_2 (Spicaria elegans) (ii) AD_3 (Mycogone I 103) to mice that had just been weaned as in the previous experiment. This time however the only parameters to be followed were behaviour, body weight and post-mortem examination.

Section 12

Preliminary toxicological evaluation of AD_2 and AD_3.

Preliminary toxicological evaluation of AD_2 and AD_3.

12.1 Experimental design

The strain of mice used in this experiment were T.O. strain. The control mice and experimental mice were composed of an equal number of males and females, 6 males and 6 females in each case, maintained in seperate cages in groups of 6. The mice were 21 days old when received, and were allowed a 3 day period on the normal diet to equilibriate before starting the experiment. The mice were then weighed, and thereafter weighed at 2 - 3 day intervals at a different time in the day relative to the last recorded weights. This was carried out in order to obtain random samples because statistical analysis, the ' t ' test was carried out on the body weights at the end of the experiment, after 11 days, and also at the end of the experiment 50% of the mice colonies in each case were sacrificed and examined for any gross anatomical changes. The individual mice were randomly selected and in each case composed of an equal number of males and females by design.

It was decided to feed the mycelia to the mice at the 25 g% level instead of the 50 g% as before, because the true protein content of AD_2 and AD_3 was not known. But also the fact that AD_2 and AD_3 may be toxic, and feeding it to the mice at the 50 g% level may result in the experiment having to be repeated at a lower level in order to obtain sufficient data.

Materials and Methods

12.2 Control diet

The control diet was powdered 41.B. containing 15 g% protein, 2.5 g% oil and 5.5 g% fibre. This was fed to the control mice in 40 g lots per day per 6 mice, that is in excess of their daily needs.

12.3 Experimental diets.

(i) 75 g% of 41.B. and 25 g% of AD_2 mycelia, (ii) 75 g% of 41.B. and 25 g% of AD_3 mycelia obtained from submerged culture in tower fermenters. These diets were fed to the appropriate mice in powder form.

Results

12.4 Post-mortems and Behaviour

There were no unnatural deaths due to the experimental diets. Post-mortems of mice sacrificed that were maintained on the experimental diets showed no observable anatomical differences compared with the control mice maintained on the normal diet. The behaviour of the mice maintained on the experimental diets compared with the controls maintained on the normal diet did not show any behavioural differences.

12.5 Statistical Analysis of Body Weights

Time in days	Diet normal		Diet AD_2 25 g%		Diet AD_3 25 g%	
	Group body weights of mice in grams.		Group body weights of mice in grams.		Group body weights of mice in grams.	
	Males	Females	Males	Females	Males	Females
0	68.3	57.0	68.5	55.0	67	57.3
3	83.0	70.5	75.5	66.5	81.5	66.5
5	97.0	86.0	94.0	83.0	101.5	80.5
7	114.5	102.5	99.5	88.0	112.0	103.0
9	120.5	107.0	102.5	88.0	118.5	101.5
11	138.0	121.5	110.5	99.0	131.0	112.5
	$x = 1165.80$		$x = 1030.00$		$x = 1132.80$	
	$\bar{x} = 97.15$		$\bar{x} = 85.83$		$\bar{x} = 94.40$	
	$n = 12$		$n = 12$		$n = 12$	
	table 1.		table 2.		table 3.	

Comparison of group body weights of mice maintained on normal diet (controls) with those maintained on normal diet containing 25 g% of AD_2 was very highly significant.

$$t = 28$$

$$^{o}_{F} = 22$$

$$p \quad 0.001$$

Therefore the experimental diet containing AD_2 is not as sufficient as the control diet.

Comparison of group body weights of mice maintained on normal diet (controls) with those maintained on a normal diet containing 25 g% AD_3 was highly significant.

$t = 2.75$

$^{o}_{F} = 22$

p 0.02 0.01

Therefore the experimental diet containing AD_3 is not as sufficient as the control diet.

Discussion and Conclusions

The experimental diets containing AD_2 or AD_3 were not adequate in terms of weight gain in the mice compared with the controls on the normal diet; nevertheless the experimental diets containing AD_2 or AD_3 were not toxic compared with the experimental diet containing AD_1 in the first experiment.

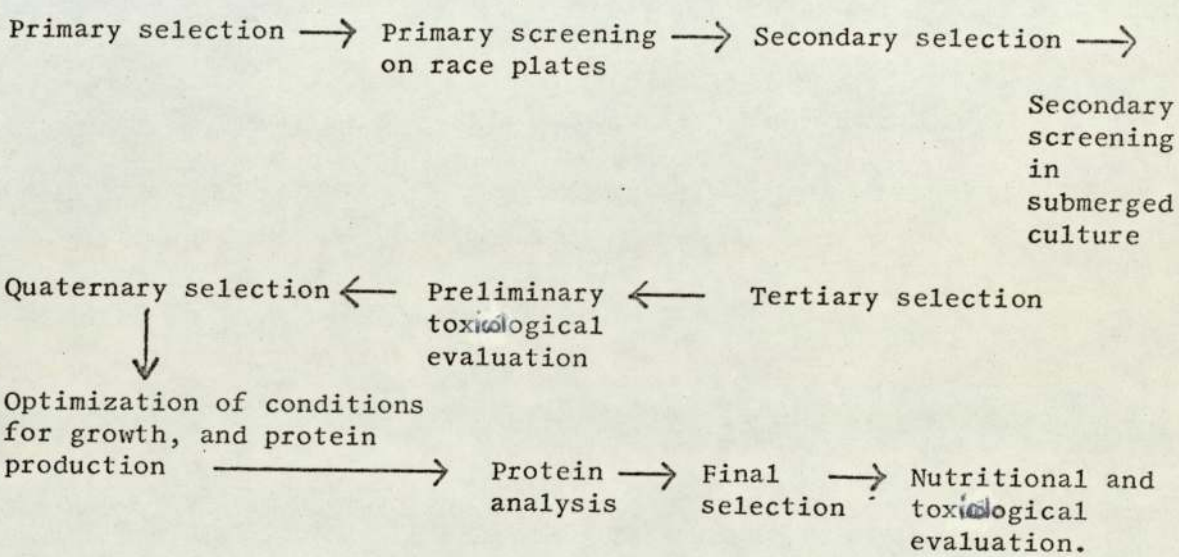
The statistical results for the experimental diet containing AD_3 gave a value of * t * = 2.75 (table 3.) which means that the difference in body weight was significant. It is most likely that if some pre-treatment is made to the mycelia of AD_3, such as disruption of the mycelial walls, it will enhance the availability of the AD_3 protein for utilization by the animals ingesting it. In fact this may apply to AD_2 or any fungi that is to be fed to mammals.

Summary of Main Discussions and Conclusions

The Basidiomycetes that were cultured successfully in submerged culture in the secondary screening, that is Poria latemarginata and Polyporus tulipferus had protein contents of 44.0 g% and 52.0 g% respectively; the total amount of protein produced in each case in 121 hours was 0.22 g% and 0.23 g% respectively. The Fungi Imperfecti selected from the secondary screening in submerged culture, that is AD_1, AD_2 and AD_3 had protein contents of 32.0 g%, 30.0 g% and 44.0 g% respectively; the total amount of protein produced in each case in 42 hours was 0.22 g%, 0.30 g% and 0.30 g% respectively. Thus there was very little difference in the total protein produced by any of these fungi. However the rate of production of the protein was greater in the Fungi Imperfecti than in the Basidiomycetes. It is obvious from this that the Fungi Imperfecti produced a larger biomass than the Basidiomycetes; thus assuming the proteins in each case to have the same biological value and availability, the animals fed on the Fungi Imperfecti would require larger quantities than if fed on the Basidiomycetes. Therefore based on this argument it is immaterial which fungus is grown for protein feed, since the advantages are cancelled out by the disadvantages in both cases. But since the rate of protein production is greater in the Fungi Imperfecti and their fermentation time is short compared with the Basidiomycetes; also the Fungi Imperfecti produce more mycelia per gram of carbohydrate utilized (yield) than the Basidiomycetes, it is obvious from these considerations that it would be more economical to use the Fungi Imperfecti selected than the Basidiomycetes. The shorter fermentation time required by the Fungi Imperfecti and their rapid rate of growth also reduce the possibilty of infections occurring in the fermenter which would normally outgrow the fungus

and colonise the substrate.

Although non-toxic fungi may be selected for protein production, this does not necessarily mean the fungi will remain non-toxic when cultured under conditions different to those found in their normal ecosystem. From the preliminary nutritional and toxological evaluation of AD_1, it is evident that it had the potential to become toxic given the right environmental conditions. Although AD_1 may not be toxic under its normal environmental conditions, certain members of its species are. Thus AD_1 must have an inherent potential to produce toxic metabolites. It is obvious that some preliminary toxicological evaluation of the fungi should be made before any attempt is made to optimize its growth conditions and before any preliminary protein analysis is made. Thus a modified screening and selection method is presented below.



The preliminary toxicological evaluation could be made by feeding the fungal mycelia mixed with the test animals normal diet at the 50% level. Obviously the mycelia must not be toxic at any level fed to the animals, but feeding it at the 50% level should allow any toxic effects to be reflected over a short duration; also

at this level the animal is less likely to suffer from malnutrition if the biological value and availability of the fungal protein is low.

If a group of fungi are thus screened and found to be non-toxic the quaternary selection may be based on the statistical analysis ('t' test) of the control animals body weights compared with the body weights of the animals maintained on the experimental diet, in both cases the animals being offered the same amount of food each day, the amount offered being determined by the control animals normal daily intake. In the preliminary toxicological evaluation of AD_2 and AD_3, the statistical analysis applied was the 't' test, the values of 't' obtained for AD_2 and AD_3 were 28 and 2.75 respectively. The smaller the value of 't' the smaller is the significant difference between two things, in this case the body weight of the mice maintained on their normal diet and the body weight of mice maintained on the experimental diets. Thus the value of 't' can be used as an index of the biological value and availability of the fungal proteins. However if a fungus contained proteins with a very low biological value, but a high availability, it is most unlikely that it will be able to compete with a fungus containing proteins of high biological value and low availability. Therefore in the extreme cases the value of 't' can be taken as an index of the biological value of the fungal proteins compared to the animals normal diet.

Since mammals do not produce chitinase the availability of the fungal proteins will normally be low, depending of course on the structure of the fungal cell walls. The majority of the protein made available to the animal will no doubt be due to leaching of the fungal cell contents, mainly from damaged fungal cells. It is

obvious therefore that when the final selection of a fungus has been made the nutritional and toxological evaluation must be based on the mycelia that had been pre-treated to enhance the availability of its proteins. This pre-treatment of the mycelia may take the form of partial digestion with hydrochloric acid, enzymic hydrolysis or mechanical disruption. Thus the enhanced availability of the fungal protein will not only lend itself to direct nutritional evaluation, but also to more reliable chemical analyses if the cell walls of the fungi are removed.

The chemical analysis of fungal proteins most employed is the Kjeldahl method. This method is somewhat inadequate, or any method which involves the total nitrogen determination to be multiplied by the factor 6.25, for this assumes that fungal proteins contain 16% nitrogen as animal proteins. In fact fungal proteins may contain more or less nitrogen than animal proteins. This total nitrogen determination not only includes amino nitrogen but also inorganic and nucleic acid nitrogen. The nucleic acid nitrogen in the case of AD_1 accounted for 8.0% of the Kjeldahl protein estimation, this is excluding the nitrogen due to the fungal cell wall constituents such as amino sugars and D-amino acids. No doubt when all these factors are considered the protein content of fungi will be found to be relatively low in many cases, but this will also apply to yeast and bacteria.

Although the protein content of many of the fungi may be found to be relatively low, the ease with which many fungi can be cultured and recovered from waste materials makes them potentially, economically viable for investigation and may provide food for thought in many areas of the world.

Appendix

Primary Screening results of the Basidiomycetes and Fungi Imperfecti not included in Secondary Selection.

Definitions and Abbreviations

Definitions

The observations made of colony growth were: radial, thin, thick, flat, domed, bushy, diffuse, dense, sparse and limited; these are defined as follows:

Thin or thick, this describes the depth of mycelia over the surface of the carob media.

Flat or domed, this describes the overall shape of the mycelia over the surface of the carob media.

Bushy, this also describes the overall shape of the mycelia over the surface of the carob media.

Diffuse or dense, this describes the distribution of mycelia over the surface of the carob media.

Sparse or limited, this describes limited colony growth in which the circular perimeter is not well defined due to irregular growth at the colonies perimeter.

Abbreviations

Flat	= F.	Thick	= Th.	Thin	= Tn.	Domed	= Do.
Dense	= D.	Sparse	= Sp.	Radial	= R.	Diffuse	= Df.
Limited	= Li.	Bushy	= Bu.				

Basidiomycetes

Polyporus palustris

Nitrogen supplements	Time	0	17	43	89	115	138	Colony growth
GA	R^2cm	0.16	0.16	0.74	3.24	5.13	6.91	Th. F. D. R.
AS		0.16	0.16	0.90	5.66	9.79	16.12	Th. F. D. R.
GA + AS		0.16	0.16	1.17	6.25	10.89	17.22	Th. F. D. R.
Unsupplemented		0.16	0.16	0.81	3.80	6.76	9.79	Tn. F. D. R.

Polyporus spraqui

Nitrogen supplements	Time	0	22	47	72	95	116	Colony growth
GA	R^2cm	0.16	0.30	0.77	1.00	2.65	4.97	Tn. F. Sp. R.
AS		0.16	0.30	0.58	1.32	2.56	4.20	Tn. F. D. R.
GA + AS		0.16	0.30	0.72	1.39	2.19	3.29	Tn. F. Sp. R.
Unsupplemented		0.16	0.30	0.60	1.00	1.56	2.30	Tn. F. Sp. R.

Trametes serialis

Nitrogen supplements	Time	0	26	72	138	Colony growth
GA	R^2cm	0.16	0.16	0.23	0.28	Df. Sp. R.
AS		0.16	0.16	0.20	0.25	Df. Sp. R.
GA + AS		0.16	0.16	0.23	0.29	Df. Sp. R.
Unsupplemented		0.16	0.16	0.25	0.25	Df. Sp. R.

Lentinus tigrinus

Nitrogen supplements	Time	0	23	47	72	96	120	Colony growth
GA	R^2cm	0.16	0.16	0.16	0.30	1.06	1.24	Tn. D. F. R.
AS		0.16	0.16	0.16	0.36	0.69	0.96	Tn. D. F. R.
GA + AS		0.16	0.16	0.16	0.38	1.03	1.78	Tn. D. F. R.
Unsupplemented		0.16	0.16	0.16	0.53	1.27	2.14	Tn. D. F. R.

Polyporus tulipferus

Nitrogen supplements	Time	0	23	47	72	119	144	Colony growth
GA	R^2cm	0.16	0.16	0.90	2.19	5.90	9.39	Th. F. D. R.
AS		0.16	0.16	0.60	1.39	4.33	6.76	Tn. F. D. R.
GA + AS		0.16	0.16	0.81	1.92	5.50	8.29	Th. F. D. R.
Unsupplemented		0.16	0.16	0.74	1.82	5.66	8.88	Tn. F. Sp. R.

Lentinus lepideus

Nitrogen supplements	Time	0	22	47	72	95	116	Colony growth
GA	R^2cm	0.16	0.16	0.30	0.58	1.60	2.60	Tn. F. D. R.
AS		0.16	0.16	0.31	0.90	1.60	2.45	Tn. F. D. R.
GA + AS		0.16	0.16	0.36	0.81	1.44	2.40	Th. F. D. R.
Unsupplemented		0.16	0.16	0.36	0.72	1.69	2.60	Tn. F. Sp. R.

Lenzites trabea

Nitrogen supplements	Time	0	17	41	66	98	114	Colony growth
GA	R^2cm	0.16	0.25	0.45	0.81	1.86	3.29	Th. F. D. R.
AS		0.16	0.16	0.35	0.60	1.20	1.63	Th. F. D. R.
GA + AS		0.16	0.16	0.35	0.60	1.76	2.30	Th. F. D. R.
Unsupplemented		0.16	0.16	0.20	0.72	1.39	1.86	Tn. F. Sp. R.

Poria monticola

Nitrogen supplements	Time	0	18	42	66	98	114	Colony growth
GA	R^2cm	0.16	0.16	0.26	0.53	1.16	1.63	Tn. Bu. Df. R.
AS		0.16	0.16	0.26	0.53	0.96	1.73	Th. Bu. D. R.
GA + AS		0.16	0.16	0.22	0.37	1.20	1.20	Th. Bu. D. R.
Unsupplemented		0.16	0.16	0.26	0.53	1.06	1.51	Tn. Bu. Sp. R.

Poria xantha

Nitrogen supplements	Time	0	17	42	65	98	114	Colony growth
GA	R^2cm	0.16	0.16	0.16	0.23	0.69	1.16	Tn. F. Sp. R.
AS		0.16	0.16	0.16	0.30	1.47	2.40	Tn. F. Sp. R.
GA + AS		0.16	0.16	0.16	0.18	0.58	0.82	Tn. F. Sp. R.
Unsupplemented		0.16	0.16	0.16	0.25	1.00	1.73	Tn. F. Sp. R.

Polyporus anceps

Nitrogen supplements	Time	0	17	43	90	115	138	Colony growth
GA	R^2cm	0.16	0.25	1.10	4.12	6.00	8.20	Tn. F. D. R.
AS		0.16	0.16	0.90	3.80	6.40	9.70	Th. F. D. R.
GA + AS		0.16	0.16	1.16	4.75	7.64	10.99	Th. F. D. R.
Unsupplemented		0.16	0.16	1.06	4.47	7.73	10.99	Tn. F. D. R.

Polyporus meliae

Nitrogen supplements	Time	0	18	43	90	115	138	Colony growth
GA	R^2cm	0.16	0.20	0.81	4.20	6.83	11.22	Tn. F. D. R.
AS		0.16	0.20	0.69	6.65	7.72	12.96	Th. F. D. R.
GA + AS		0.16	0.40	1.10	5.66	9.18	14.44	Th. F. D. R.
Unsupplemented		0.16	0.20	0.69	3.72	6.37	10.63	Tn. F. Sp. R.

Poria nigra

Nitrogen supplements	Time	0	27	53	78	101	122	Colony growth
GA	R^2cm	0.16	0.16	0.66	1.39	2.45	3.61	Th. F. D. R.
AS		0.16	0.16	0.72	1.73	3.06	4.69	Th. F. D. R.
GA + AS		0.16	0.16	0.60	1.24	2.56	3.24	Th. Do. D. R.
Unsupplemented		0.16	0.16	0.57	1.04	1.86	2.56	Th. F. D. R.

Poria oleracea

Nitrogen supplements	Time	0	21	46	71	94	116	Colony growth
GA	R^2cm	0.16	0.25	0.39	0.69	1.28	1.90	Th. F. D. R.
AS		0.16	0.25	0.46	0.90	1.96	2.50	Th. F. D. R.
GA + AS		0.16	0.25	0.53	1.00	1.50	2.56	Th. F. D. R.
Unsupplemented		0.16	0.25	0.42	0.81	1.44	2.10	Tn. F. Sp. R.

Hirschioporus abietinus

Nitrogen supplements	Time	0	26	72	138	Colony growth
GA	R^2cm	0.16	0.16	0.36	1.10	Tn. Sp. R.
AS		0.16	0.16	0.29	0.90	Tn. Sp. R.
GA + AS		0.16	0.16	0.46	1.10	Tn. Sp. R.
Unsupplemented		0.16	0.16	0.30	0.59	Tn. Sp. R.

Polyporus versicolor

Nitrogen supplements	Time	0	18	43	89	115	138	Colony growth
GA	R^2cm	0.16	0.25	0.77	3.47	5.66	8.49	Tn. F. Sp. R.
AS		0.16	0.25	0.68	3.53	6.33	9.48	Th. F. D. R.
GA + AS		0.16	0.30	0.90	4.40	7.37	10.75	Th. F. D. R.
Unsupplemented		0.16	0.16	0.90	4.20	6.91	10.43	Tn. F. D. R.

Poria latemarginata

Nitrogen supplements	Time	0	23	47	54	71	75	Colony growth
GA	R^2cm	0.16	0.96	4.84	6.50	12.71	14.29	Th. F. D. R.
AS		0.16	0.87	4.88	6.50	13.43	14.94	Th. F. D. R.
GA + AS		0.16	1.21	6.40	8.50	15.84	17.64	Th. F. D. R.
Unsupplemented		0.16	0.96	6.00	7.92	15.72	17.05	Tn. F. Sp. R.

Poria placenta

Nitrogen supplements	Time	0	26	72	138			Colony growth
GA	R^2cm	0.16	0.16	0.31	1.28			Bu. Sp. R.
AS		0.16	0.16	0.25	0.46			Bu. Sp. R.
GA + AS		0.16	0.16	0.25	0.81			Bu. Sp. R.
Unsupplemented		0.16	0.16	0.20	0.49			Bu. Sp. R.

Fungi Imperfecti

Mycogone I 103

Nitrogen supplements	Time	0	21	43	67	92	115	Colony growth
GA	R^2cm	0.16	0.25	1.00	1.85	2.49	4.24	Th. Do. D. R.
AS		0.16	0.25	0.81	1.23	1.46	1.82	Tn. Do. D. R.
GA + AS		0.16	0.30	1.14	2.13	5.29	7.02	Th. Do. D. R.
Unsupplemented		0.16	0.23	0.81	1.34	2.40	3.24	Tn. Do. D. R.

Cephalosporium I 87

Nitrogen supplements	Time	0	21	42	67	91	115	Colony growth
GA	R^2cm	0.16	0.16	0.22	0.46	0.86	1.24	Tn. F. D. R.
AS		0.16	0.16	0.16	0.23	0.29	0.40	Tn. F. Li. R.
GA + AS		0.16	0.16	0.25	0.69	1.13	1.72	Tn. F. D. R.
Unsupplemented		0.16	0.16	0.16	0.32	0.42	0.56	Tn. F. Li. R.

Dendrophoma I 57

Nitrogen supplements	Time	0	21	42	67	92	115	Colony growth
GA	R^2cm	0.16	0.31	1.20	2.25	2.92	3.92	Th. Do. D. R.
AS		0.16	0.21	0.71	1.10	1.23	1.51	Tn. Do. D. R.
GA + AS		0.16	0.33	0.96	2.16	2.62	3.84	Th. Do. D. R.
Unsupplemented		0.16	0.21	0.74	0.94	1.20	1.44	Tn. F. Sp. R.

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Publications

THE PRODUCTION OF FUNGAL BIOMASS IN AEROBIC TOWER FERMENTERS

by R.N.Greenshields, G.G. Morris, B. Daunter, R. Alagaratnam and F.K.E.Imrie.

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as presented at the First International Mycological Congress, Exeter, September 1971.

1. The tower or tubular fermenter can be described as an elongated non-mechanically stirred fermenter with an aspect ratio (height to diameter ratio) of at least 6:1 on the tubular section or 10:1 overall through which there is a uni-directional flow of medium or gases[1]. It can be used for batch, semi-continuous and continuous fermentations and can be classified using Herberts' criteria[2] of continuous systems, as a partially closed heterogenous tubular system. This was clearly demonstrated when this type of fermenter and its associated system was successfully used for the continuous production of ale and lager beer on laboratory[3,4], pilot[5] and commercial scales[6,7,8]. Such application including that when applied to the similar fermentations of cider, wine, whiskey-wash and vinegar charging-wort are growth-associated metabolite fermentations involving yeast under anaerobic or partially aerobic conditions. Its application in a fully aerobic situation has obviously been considered. Initial work has indicated that the aerobic conversion of ethanol to acetic acid by acetic acid bacteria in vinegar manufacture is feasible and continuous runs have been obtained on a laboratory scale[8,9,10,11]. Moreover, the production of yeast biomass in a flocculent condition is also possible on batch and semi-continuous systems and have been accomplished on laboratory, pilot and commercial scale[8,12]. This process is also being considered for non-flocculent yeast in a continuous system.

The work now presented here is the extension of this application to the filamentous fungi, particularly for the production of biomass - the production of fungal metabolites has been considered[8] but is the subject of further study and will not be dealt with here. The fermenter design[9] is shown in Diagram I. This does not materially differ from that used for anaerobic fermentations[8] except that (i) a means for aeration is provided at the bottom of the tower, (ii) the expansion chamber and thus the air/fob space is larger, (iii) some means is provided to prevent foaming and (iv) if continuous, the overflow and separator is of a different design.

The experimental fermenter vessels were made of glass, high density polythene, polypropylene or stainless steel and were sterilised both chemically (available chlorine) and by steam (up to 5 p.s.i). The sinters were made of glass, P.T.F.E., sintered stainless steel and occupied the whole fermenter tube cross-section and thus effectively held up the fermentation liquid.[8] For larger vessels a simpler aerator was used made of stainless steel and which sat on the bottom of the fermenter.

Four sizes of fermenter were used:-

(a) Experimental tubes 1 to 5 litre. 1'0" to 4'0" x 3" diameter.

(b) Laboratory scale (small) 10 to 20 litre. 4'0" to 8'0" x 4" diameter.

(c) Laboratory scale (large) 50 litre 12'0" x 6" diameter.

and (d) Pilot scale 500 to 1000 litre 15'0" to 25'0" x 1'0" diameter.

2. The fungi were grown in the tower fermenters on two media - one based on a supplemented aqueous extract of carob bean (Ceratonia siliqua) and the other based on supplemented molasses.

Normally appropriate quantities of nitrogen sources were added in the form of inorganic ammonium salts or as glutamic acid to give the required Carbon : Nitrogen ratios.

Some preselection of the fungi was made using necessary criteria to ensure the most economic and suitable biomass production. This involved:

(i) A primary selection based on literature survey using the following criteria:

(i) Protein content

(ii) Protein extract

(iii) Toxicity

(iv) Growth rates

(v) Calorific value

(vi) CHO conversion efficiency

followed by:

(ii) A primary screening using race plates from which growth rates were ascertained,

then (iii) A secondary selection using experimental tubular fermenters.

Out of some forty fungi selected, the Fungi imperfecti appeared to grow best. - Table I.

The Basidiomycetes were slower growing and although several were tried the results were not promising. It was possible that the nutritional requirements were not satisfied by the media used. The results are shown in Table 2 together with those typical of the Phycomycetes tested. The phycomycetes grew easily, but biomass yields were too low for further consideration of economic biomass production.

3. It was decided to make a detailed study of the batch growth of Aspergillus niger in various sizes of tower fermenter to ascertain its growth kinetics in terms of (a) parameters which prescribe the fermenter, and (b) parameters which prescribe the medium in an attempt to establish the optimum for economic biomass production in terms of growth rate, yield, protein content and carbohydrate conversion efficiency.

(a) Parameters which prescribe the fermenter.

(i) Aeration rate

Preliminary experiments in laboratory scale towers (10 litre) were performed to determine optimum conditions of aeration and agitation relative to the volume of air passed through the fermenter. In addition the optimum height to diameter ratio was calculated from this data. In tower fermenters oxygen transfer and agitation is caused by the air itself. Within the limits of the fermenters used, oxygen transfer efficiency and agitation increased linearly with aeration rate. Oxygen transfer rates were determined using the sulphite oxidation method of Cooper and co-workers which measured the maximum rate of solution when dissolved oxygen concentration is very nearly zero.

Graph 1 showed that an aeration rate of between 1.5 to 2 volumes of air per volume of liquid are near the optimum for oxygen transfer in this fermenter.

It should be noted that in laboratory scale fermenters it is impractical to use this optimum aeration rate and values of just below 1 vol/vol/min are generally used.

(ii) Aspect ratio

The aspect ratio of the laboratory scale tower (10 litre, 9 cm. diameter) was changed by varying the height of the liquid and hence its volume, whilst keeping the aeration rate per volume of liquid constant. Graph 2 shows that using these conditions, it can be seen that an aspect ratio of 16:1 appears to be optimal for a tower diameter of approximately 9.0 cm.

(iii) Foaming

It has been observed that during actual fermentation runs the addition of an antifoam agent had a deleterious effect on the dissolved oxygen concentration within the fermenter[11]. It caused coalescence of the air bubbles making mass transfer of oxygen into solution far less effective. The 'antifoam effect' was greater with greater mould concentrations, a stage being reached where its effect was irreversible. For this reason, a mechanical foam breaker was fitted into the expansion chamber of the fermenter, obviating the use of antifoam. This resulted in a 75%

increase in the dry weight.

(b) Parameters which Prescribe the Medium

Under submerged conditions the morphology of A.niger varies from diffuse growth through a range of pellet sizes up to several millimetres in diameter[14]. Control of a particular morphology was found to be advantageous. The tower fermenter facilitated this control, unlike a stirred fermenter, it presents relatively little shear force to disrupt the colony form. The morphological form of the organism used was selected by calculating (1) Respiratory demands and requirements; (2) Viscosity of the fermentation broth and (3) Oxygen transfer into the fermentation broth.

The mould morphology within the tower fermenter is dictated largely by the form and size of the inoculum used, secondly by the degree of agitation a few hours after seeding the fermenter and thirdly by the method of preparation of the inoculum.

Graph 3 shows the relationship between the size of pellet and the spore inoculum used.

The respiratory demands and requirements of the organism are at present being studied. It must be ascertained whether the oxygen is breaking down carbohydrate to be channelled into biomass production alone or whether there is an accumulation of metabolites. It is suggested therefore that a particular morphology may be optimal for both oxygen consumption and carbohydrate utilisation. Graph 4 shows the decline in oxygen uptake per gram of mould in relation to the size of the colonies. By computing the maximum growth rates together with oxygen consumption for the organism an optimum figure for pellet size may be calculated for particular circumstances and particular fermentation vessels.

Experiments concerning the viscosity of a fermentation broth have shown a relationship between the degree of viscosity and the size of pellet formed. It can be seen from Graph 5 that there will be less energy required to agitate a broth containing descrete pellets as there is for a diffuse mycelial culture. It is also true to say that a

viscous broth causes a greater tendency towards coalescence of air bubbles, thus creating a situation where it might be possible to aerate a broth containing pellets more efficiently than it is to aerate a broth with filamentous organism.

The optimum media conditions and constituents for the production of cell biomass and protein content were determined experimentally for A. niger, the basis of the media being beet molasses which was supplemented where necessary by ammonium sulphate as a nitrogen source and sodium dihydrogen phosphate as the source of phosphate.

(i) Carbohydrate concentration:

It was found that A. niger exhibited a wide tolerance to sugar concentration. Graph 6 shows that there is no marked change in growth rate between 5 and 12 g%. The apparent optimum lay between 7 and 8 g% However, due solely to economic consideration a slightly lower concentration might be advantageous when considering water and effluent factors. The protein concentration of the organism did not vary significantly over this optimum range of carbohydrate concentration.

(ii) Carbon-Nitrogen ratios:

Supplementation of a 5 g% carbohydrate media by various quantities of ammonium sulphate was made in order to vary the C:N ratio. Graph 7 illustrates the change in growth rates and protein contents during these experiments. The optimum growth rate lay between 7:1 and 13:1 whilst the optimum protein content is at 6:1. At higher C:N ratios however a great deal of fat was seen to be deposited in the mycelium.

(iii) Temperature:

The range of temperature studied was 20 to 40°C. Graph 8 shows that there is an optimum value for growth rate between 27°C and 33°C for the A. niger. Of economic importance is the fact that at elevated temperatures the fungal metabolism is increased, giving rise to a greater utilisation of carbohydrate. However, this may not necessarily be channelled into biomass production.

This study gives an indication of the behaviour of A.niger in a tower fermenter but can only provide a useful guide to the behaviour of any other filamentous fungus in such a vessel. It should also be pointed out that the medium is also unique in this respect and the use of molasses medium for this study can only give a guide to other organism on other media.

Scale. As previously indicated aerobic tower fermenters were constructed from laboratory up to pilot scale. Preliminary experiments have shown that the findings in the 5 litre laboratory scale tower fermenters were applicable on a 10x scale-up in fermenter size (i.e. volume) to 50 litre and again on a further 10 or 20x scale-up in fermenter size to 500 and 1000 litre. Moreover the design of such fermenter vessels could be simplified enabling cheap polypropylene fermenters to be used which gave high concentrations of mould biomass economically. If, therefore, fungal biomass is required for whatever purposes, food, protein, biochemicals or enzymes, in developing countries, then a cheap fermenter is required. The tower fermenter is such a fermenter because of its simplicity of design and construction, its advantageous kinetics and its flexibility.

DIAGRAM 1

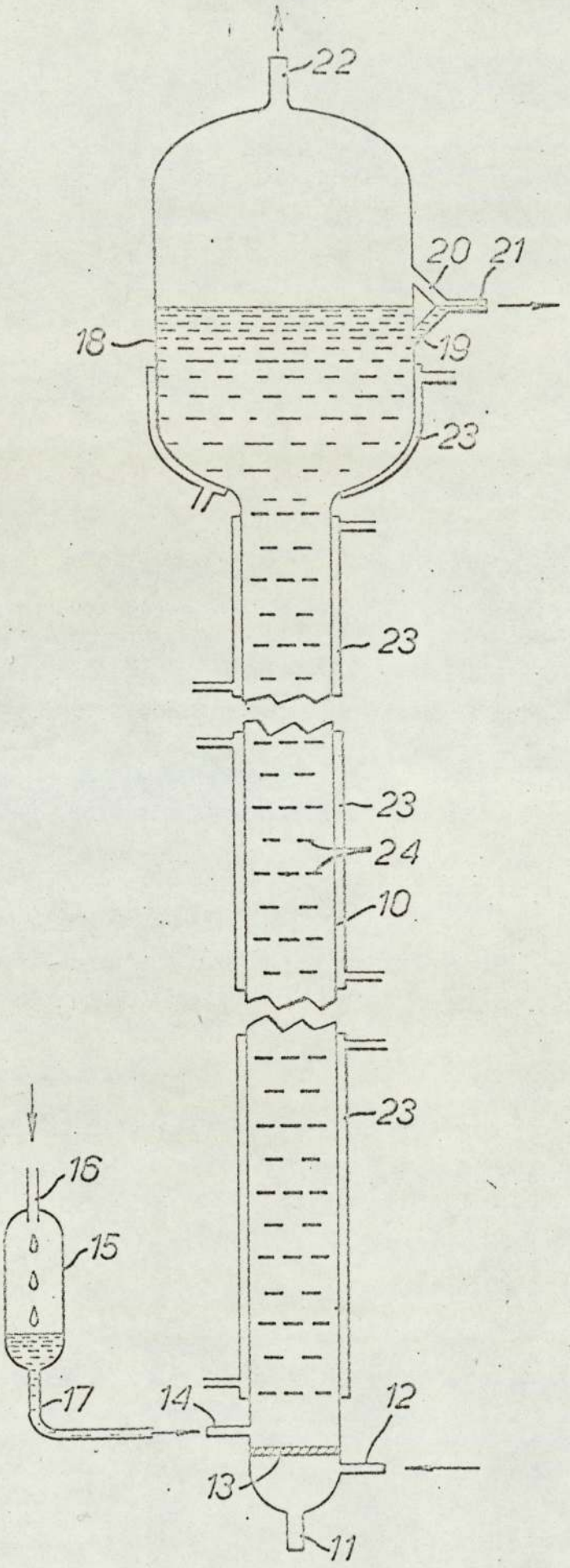


TABLE 1 - GROWTH OF FUNGI IMPERFECTI IN TOWER FERMENTERS

Organism		Medium	Scale (l)	Time maximum yield (hr)	Yield (g%)	Protein (g%)
☐ 1. *Aspergillus luchuensis*	(RA)	Molasses	50	112	1.62	42.7
☐ 2. *Aspergillus niger*	(RA)	Molasses	50	90	1.20	37
3. *Aspergillus oryzae*	(RA)	Molasses	50	120	0.87	34.0
4. *Aspergillus sydowi*	(RA)	Molasses	5	40	0.75	43.5
☐ 5. *Cladosporium cladosporoides*	(BD)	Carob	1	42	1.0	30 - 36
☐ 6. *Cladosporium cladosporoides*	(RA)	Molasses	5	30	0.72	36.5
7. *Cochliobolus sativus*	(BD)	Carob	1	64.8	0.51	35.35
8. *Fusarium oxysporum*	(RA)	Molasses	5	42	1.29	52.1
9. *Heterocephalum aurantiacum*	(RA)	Molasses	5	44	0.61	38.5
10. *Mycogone spp.*	(BD)	Molasses	1	41.9	0.70	45.9
☐ 11. *Penicillium chrysogenum*	(RA)	Molasses	50	72	1.2	36.0
☐ 12. *Penicillium notatum*	(RA)	Molasses	50	96	1.2	39.2
☐ 13. *Spicaria elegans*	(BD)	Carob	1	40	1.0	30

TABLE 2 -- GROWTH OF BASIDIOMYCETES IN TOWER FERMENTERS

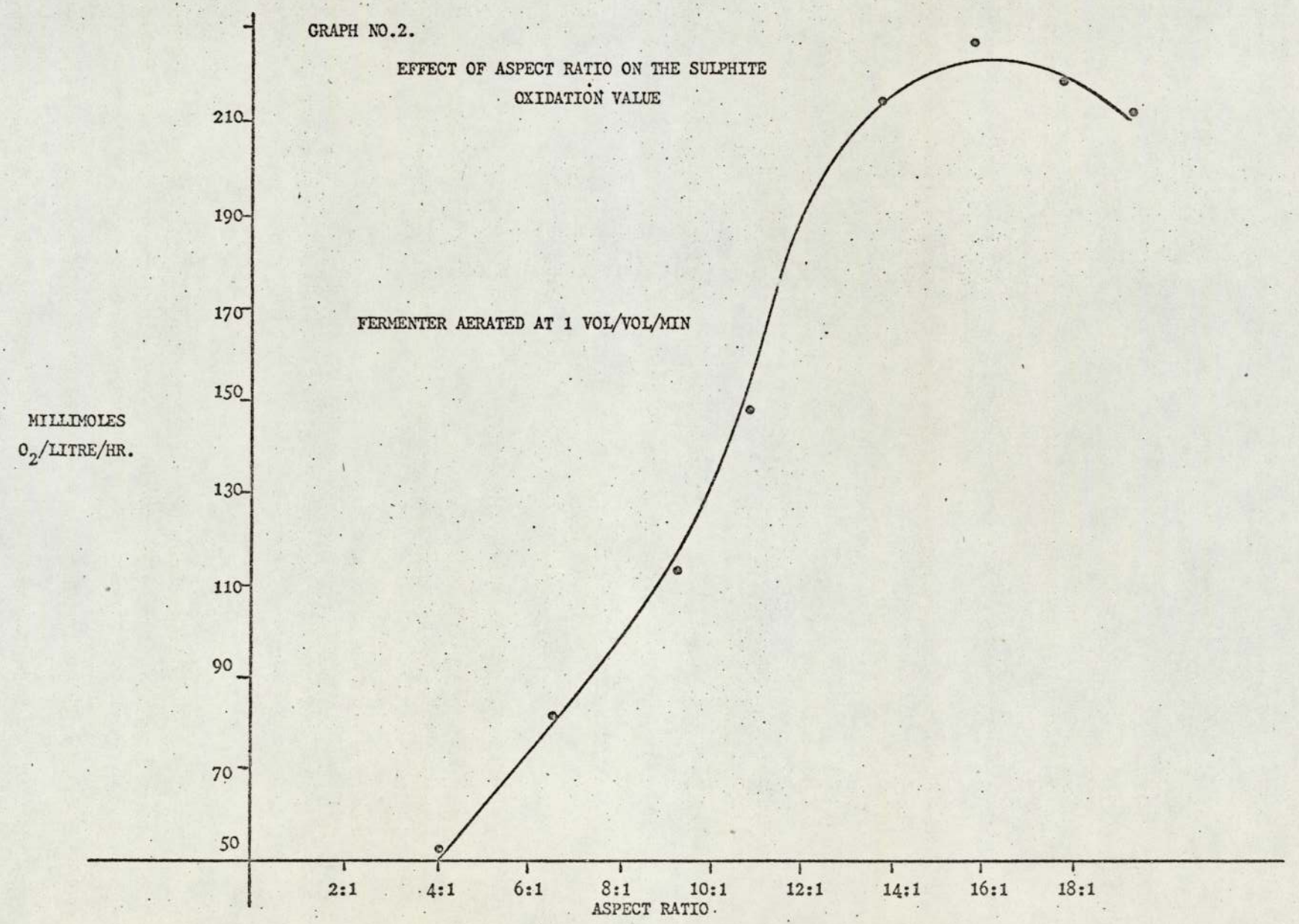
	Organism		Medium	Scale (l)	Time maximum yield (hr)	Yield (g%)	Protein (g%)
1.	*Lenzites trabea*	(RA)	Molasses	2	96	0.67	53.2
2.	*Polyporus tulipferus*	(BD)	Carob	1	121	0.58	44.01
3.	*Poria latemarginata*	(BD)	Carob	1	121	0.55	51.75

GROWTH OF PHYCOMYCETES IN TOWER FERMENTERS

	Organism		Medium	Scale (l)	Time maximum yield (hr)	Yield (g%)	Protein (g%)
1.	*Mucor racemosus*	(RA)	Molasses	50	90	0.94	43.4
2.	*Rhizopus oligosporus*	(RA)	Molasses	50	76	0.65	39.2
3.	*Rhizopus orynae*	(RA)	Molasses	5	42	0.72	38.6

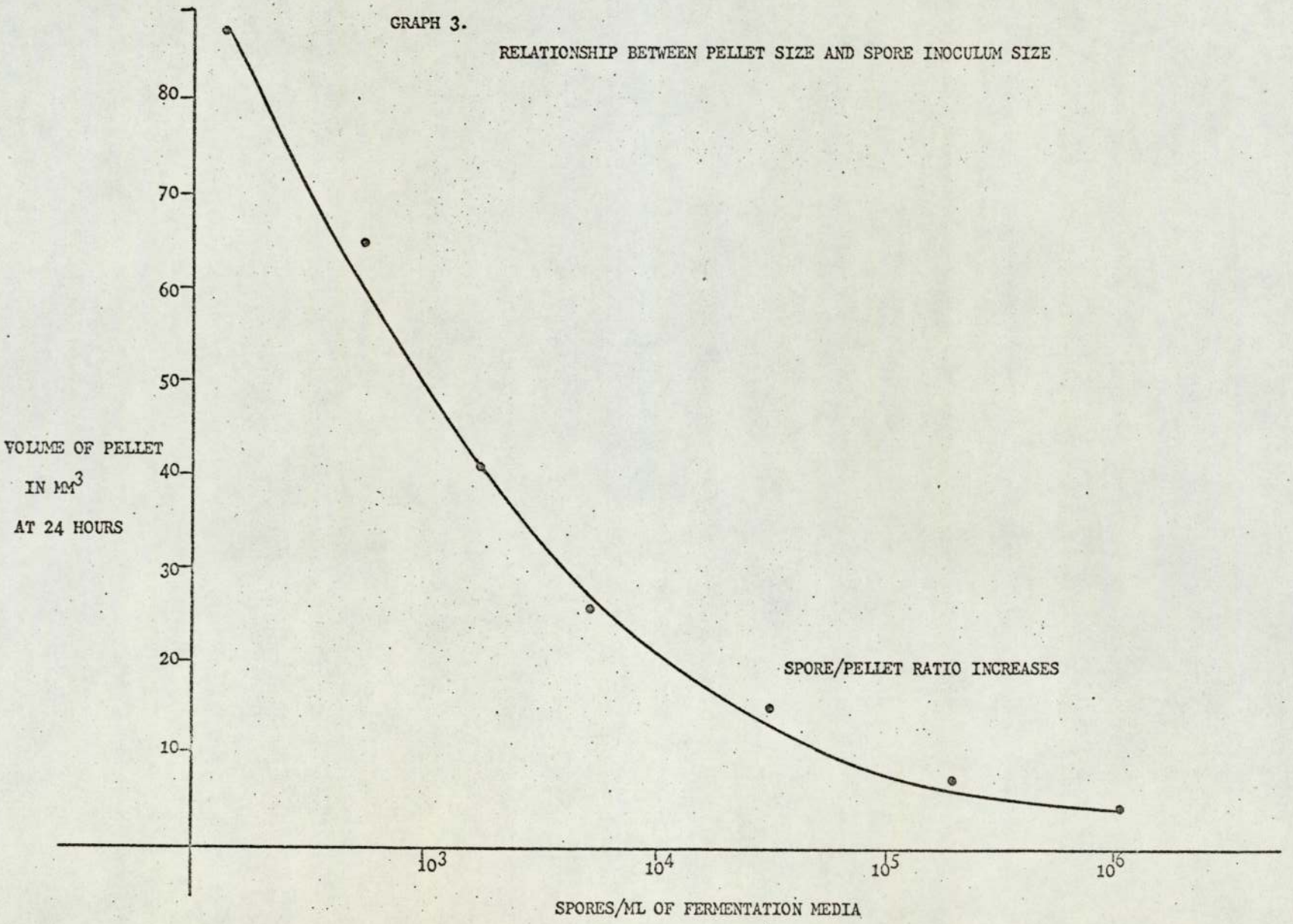
GRAPH NO.2.

EFFECT OF ASPECT RATIO ON THE SULPHITE OXIDATION VALUE



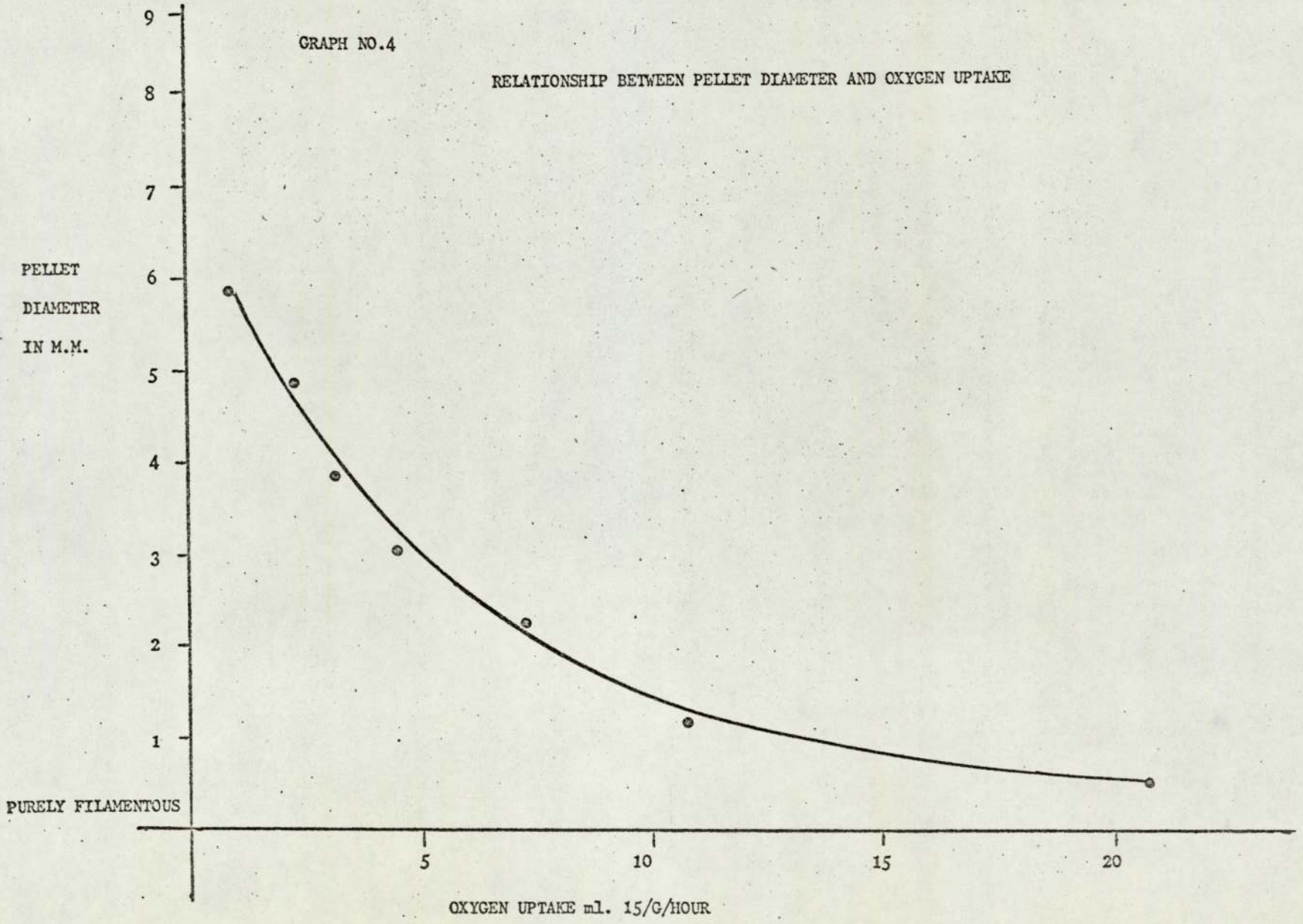
GRAPH 3.

RELATIONSHIP BETWEEN PELLET SIZE AND SPORE INOCULUM SIZE

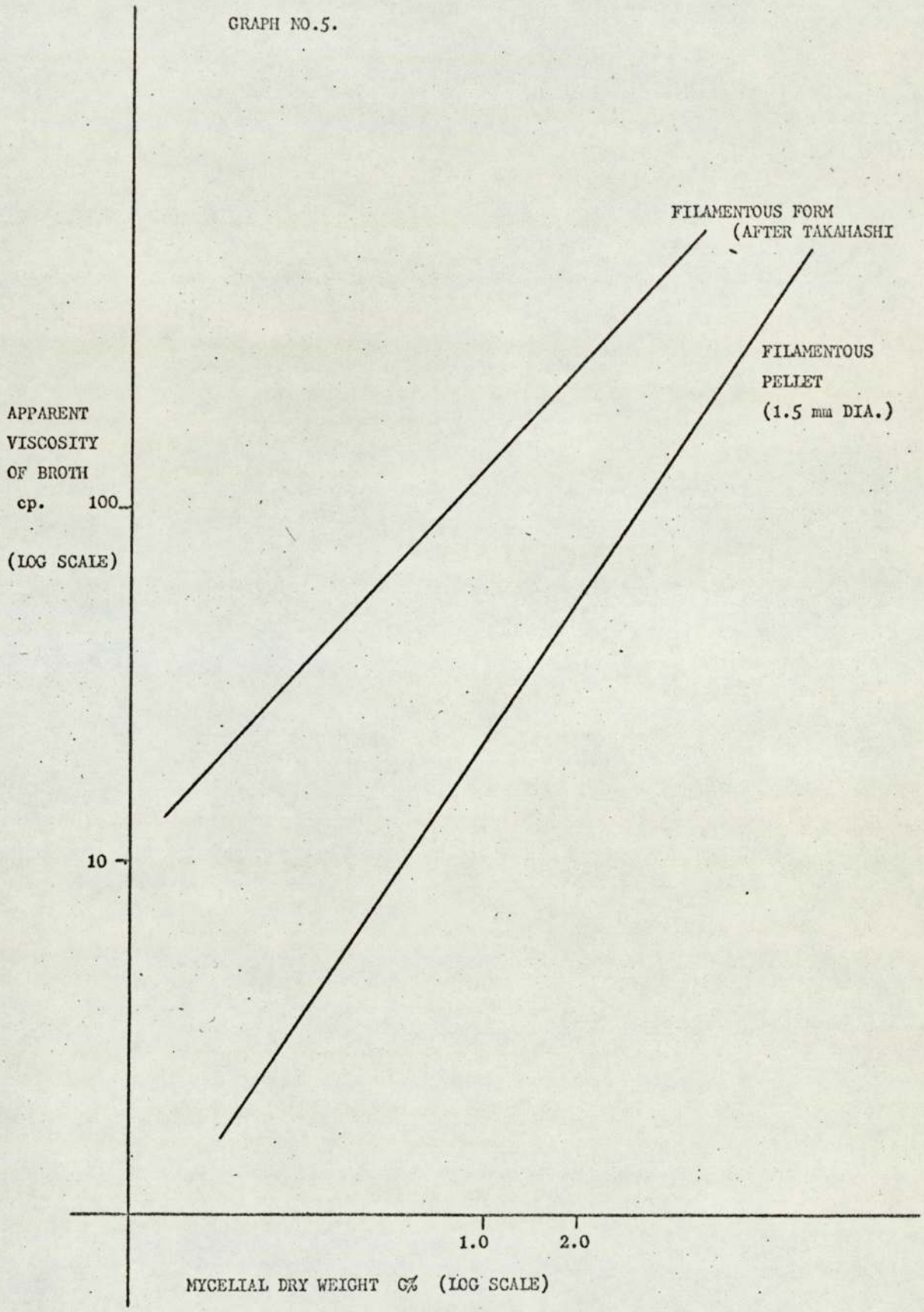


GRAPH NO.4

RELATIONSHIP BETWEEN PELLET DIAMETER AND OXYGEN UPTAKE

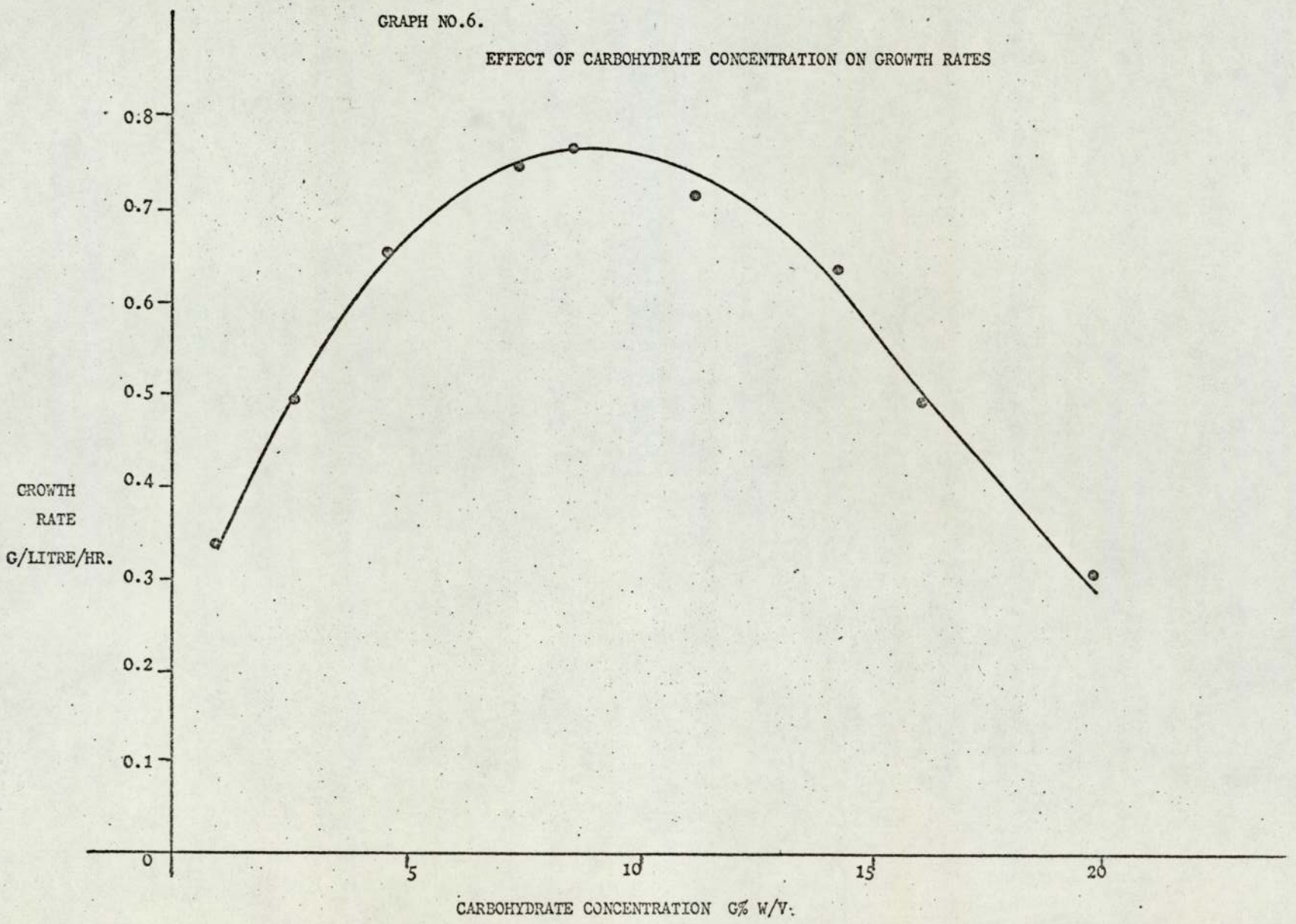


GRAPH NO.5.


FILAMENTOUS FORM
(AFTER TAKAHASHI
FILAMENTOUS
PELLET
(1.5 mm DIA.)
APPARENT
VISCOSITY
OF BROTH
cp.
100
(LOG SCALE)
10
1.0
2.0
MYCELIAL DRY WEIGHT C% (LOG SCALE)

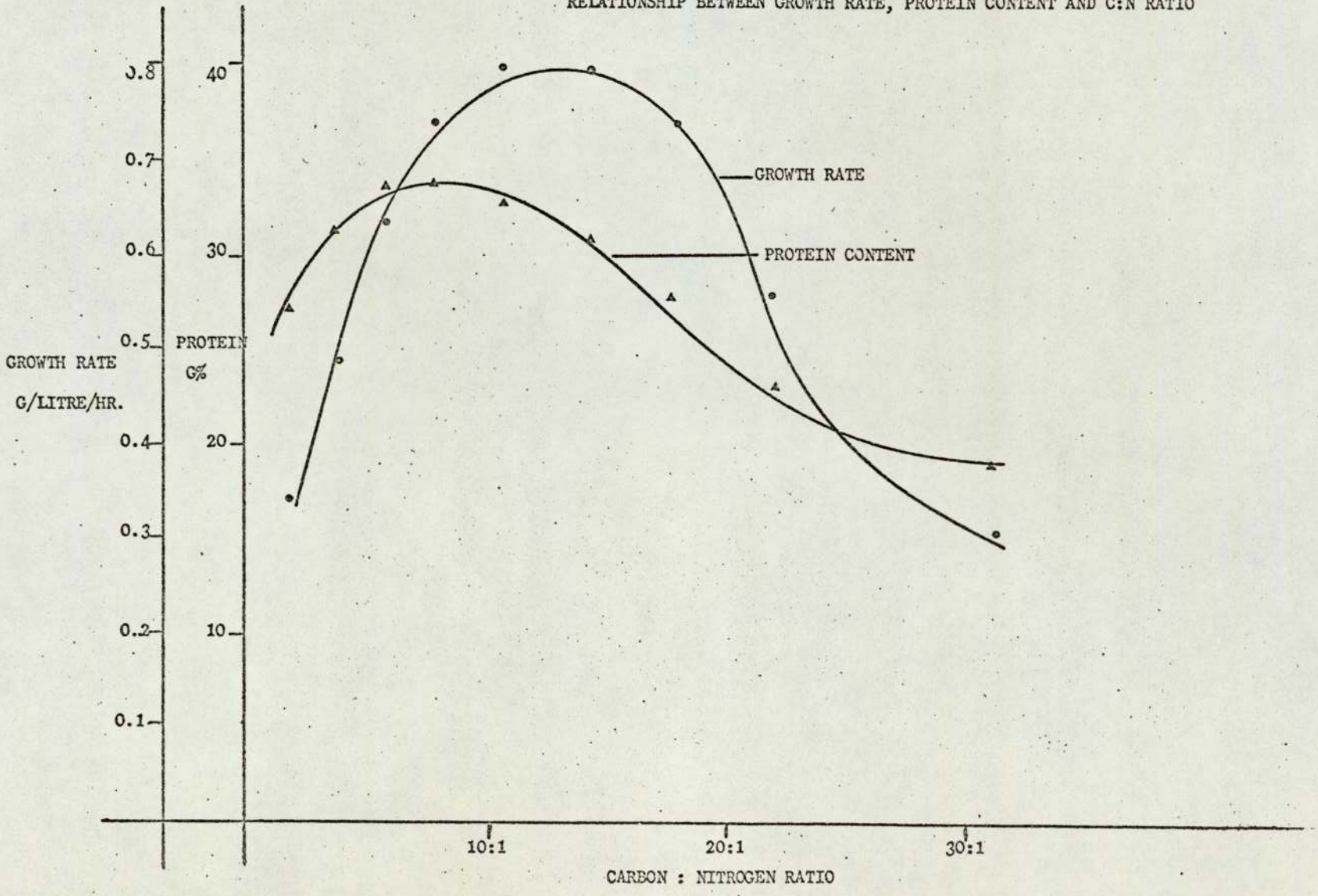
GRAPH NO.6.

EFFECT OF CARBOHYDRATE CONCENTRATION ON GROWTH RATES



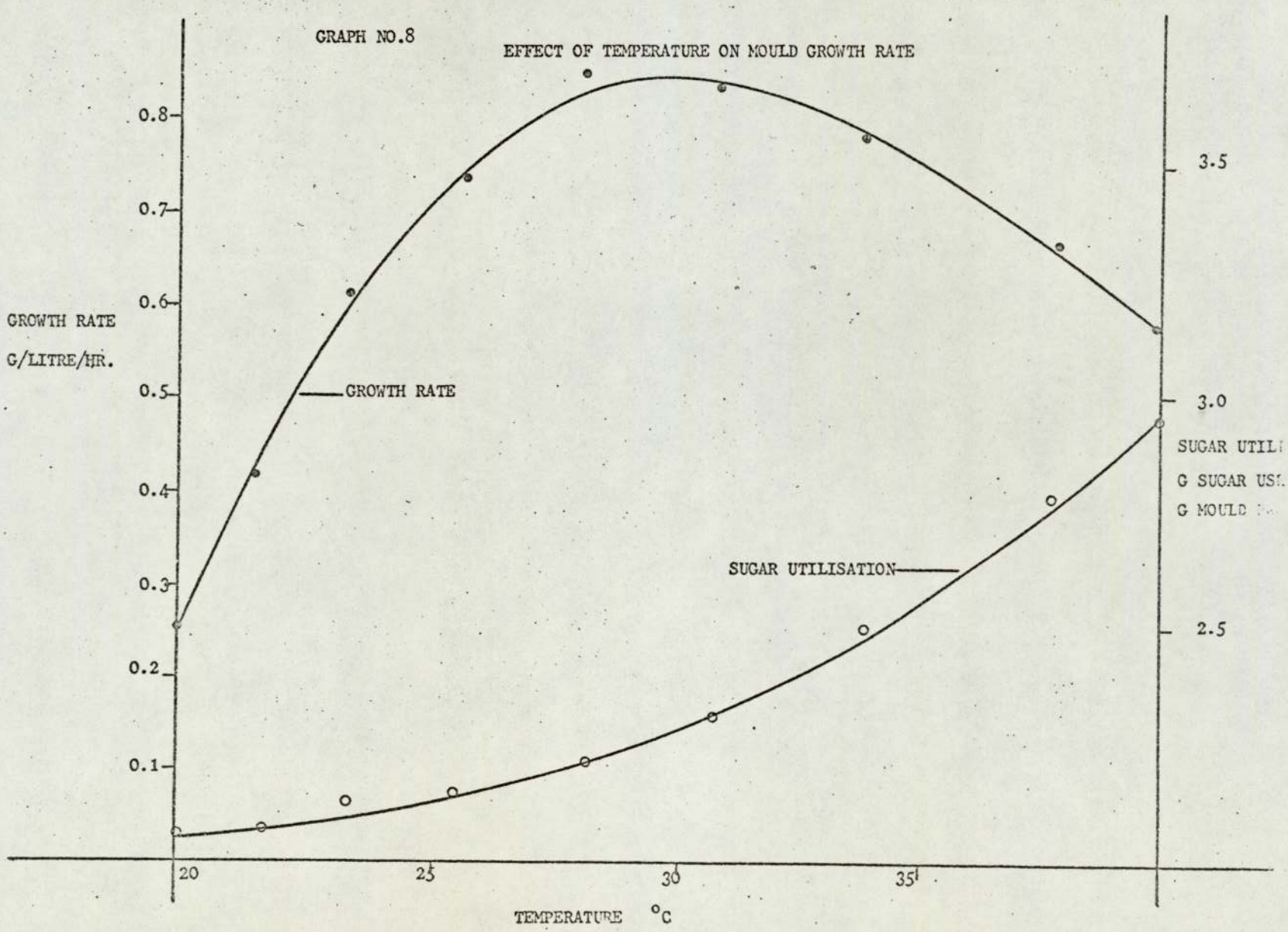
GRAPH NO.7

RELATIONSHIP BETWEEN GROWTH RATE, PROTEIN CONTENT AND C:N RATIO



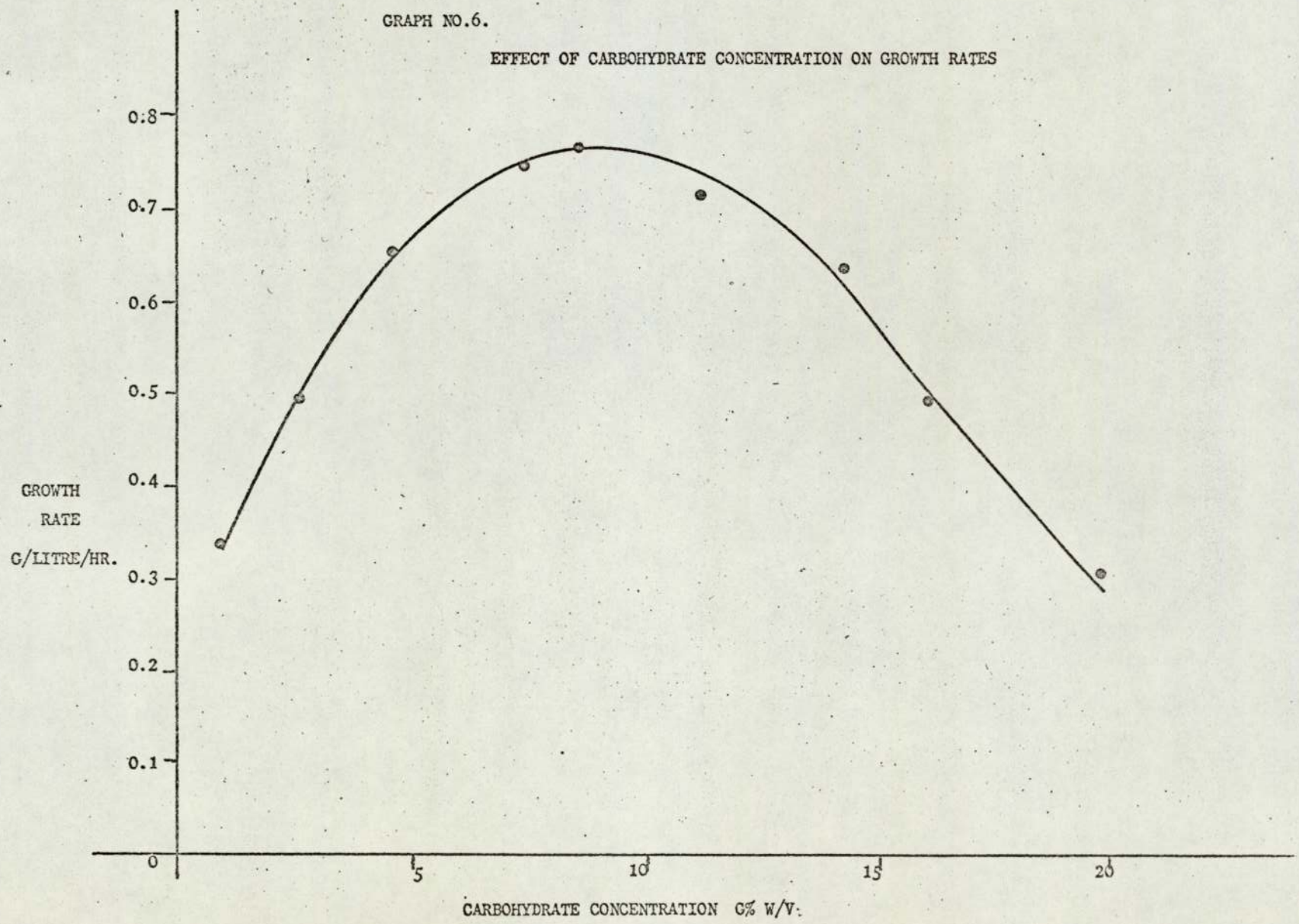
GRAPH NO.8

EFFECT OF TEMPERATURE ON MOULD GROWTH RATE



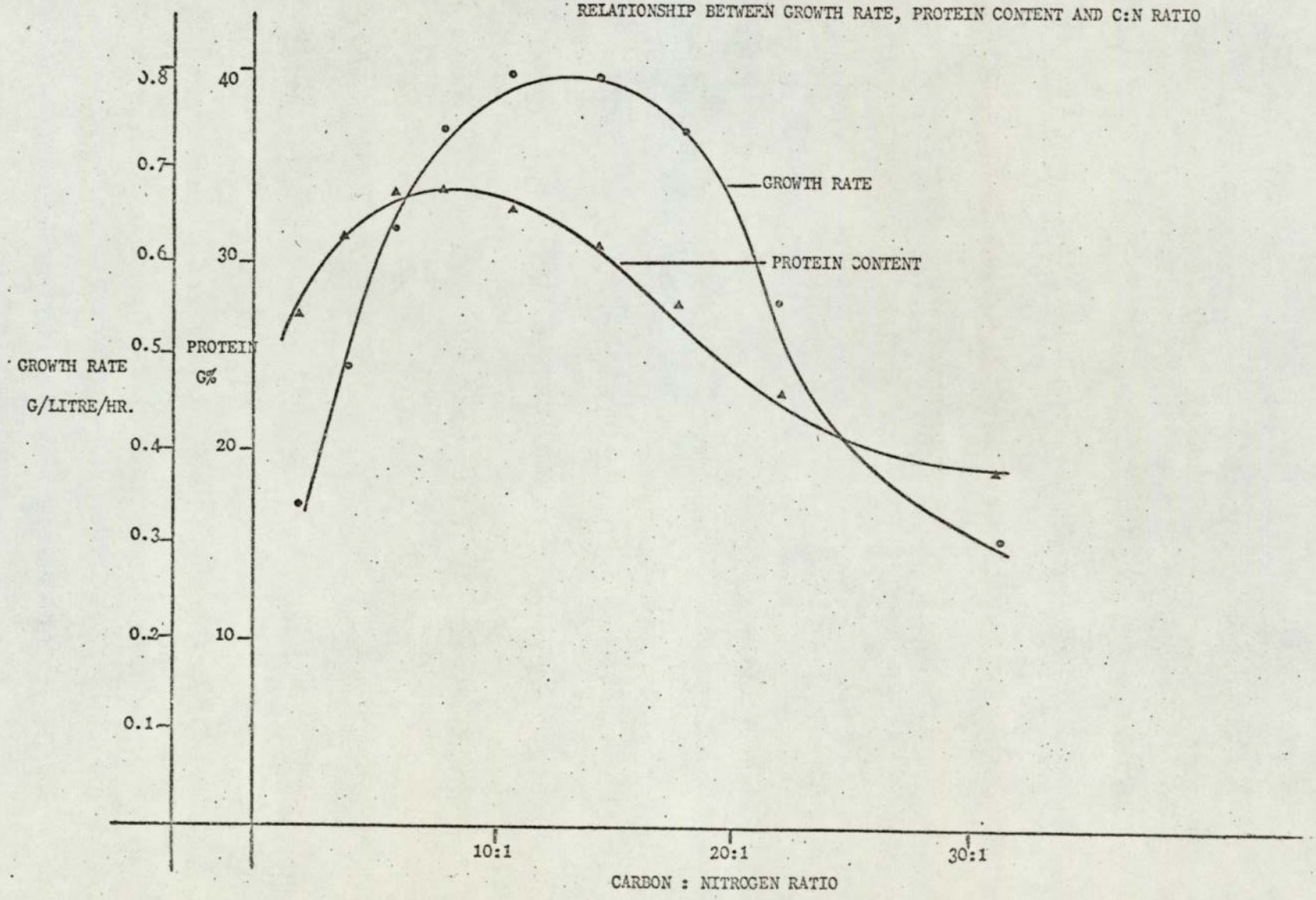
GRAPH NO.6.

EFFECT OF CARBOHYDRATE CONCENTRATION ON GROWTH RATES



GRAPH NO.7

RELATIONSHIP BETWEEN GROWTH RATE, PROTEIN CONTENT AND C:N RATIO



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THE GROWTH AND MORPHOLOGY OF MICROORGANISMS IN TOWER FERMENTERS

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General Paper Session - 12 Mass Transfer. Paper 11.

24th March 1972

Mr. Chairman - Ladies and Gentlemen.

The tower fermenter has been described[1] as an elongated non-mechanically stirred vessel with a high aspect ratio containing micro-organisms suspended in medium and through which there is a uni-directional flow of medium and/or gases.

SLIDE 1 shows the basic designs of an aerobic[2] and an anaerobic[3] tower fermenter with aspect ratios (height to diameter ratio) of at least 6:1 on the tubular section or 10:1 overall.

Such fermenters have been used for batch, semi-continuous and continuous fermentations on laboratory and pilot-scales both for the production of metabolites and biomass[4,5]. Moreover, their successful application has been made on commercial scale for the production of ale, lager, and vinegar charging-wort[5,6,7,8]. SLIDE 2a b.

A variety of industrially important micro-organisms (yeasts, moulds and bacteria) have therefore been cultured in tower fermenters under aerobic or microaerophilic conditions on a variety of media and their morphological form and growth characteristics studied.

Since the mixing and agitation in tower fermenters is accomplished by the aeration and/or release of fermentation gases, relatively little shear

force occurs to disrupt the form of the micro-organism. A colony form reflecting the micro-morphology of the organism develops which is markedly influenced by the various fermentation conditions.

Most micro-organisms grow in the form of aggregates rather than diffuse growth. Single-celled or chain-forming organisms formed flocs or compact colonies whilst filamentous types gave various forms of pellets depending on their concentration and culture conditions.

Brewing yeasts, Saccharomyces spp., in continuous culture could be divided into three groups, (I) non flocculent, (II) flocculent, depending on medium concentration and dilution rate and (III) flocculent independent of medium concentration and dilution rate. Saccharomyces cerevisiae Type I yeasts rarely attained more than 3 g% w/v expressed as centrifuged wet weight in the tower whether under aerobic or micro-aerophilic conditions and are rapidly washed from the tower at all dilution rates in continuous culture. SLIDE 3 illustrates the light aggregation found. Whereas under brewing conditions (micro-aerophilic), type II yeasts (called physically-limited yeasts) gave light powdery flocs up to 0.3 cm. diameter and had concentrations up to 25 g% w/v. Sedimentation of these flocs gave the heterogeneity essential to the fermentation characteristics of beer fermentation. At any particular specific gravity of the medium there is a critical volumetric efficiency (space-velocity i.e. volume of wort per unit time per fermenter volume) which if exceeded causes a complete wash-out of the organism. This critical speed is dependent on yeast strain, fermentation temperature and media composition. SLIDE 4 and 5 shows typical flocs of this yeast type, whilst SLIDE 6 shows the microscopic character of the floc (40% free space) and SLIDE 7 the appearance in the fermenter. Type III on the other hand, fermentation limited yeasts attained higher concentrations (25 - 45 g% w/v) having heavy 'sticky' flocs up to 1.3 cm. diameter which often aggregated into massive clumps or plugs. These are retained at all but very high volumetric efficiencies but at any particular specific gravity of the medium, the fermentation efficiency is dependent on volumetric efficiency. SLIDE 8 shows the microscopic character of the floc, the yeasts are somewhat compressed and have only 25% free space.

SLIDE 9 shows the appearance of the yeast in the fermenter. Saccharomyces carlsbergensis (lager yeasts) gave characteristic flocs which varied between type II and type III forms depending on temperature conditions, the volumetric efficiency and the fermentation efficiency. The Type II form appears generally at the early stage of fermentation whereas the Type III form appears later. SLIDE 10 exhibits both types.

In aerobic culture for biomass production, Saccharomyces spp. of the type II and III forms grew as compact erythrocyte-shaped colonies which could grow up to 1.0 cm. in diameter. These forms were very friable but gave cell concentrations on settling of up to 85 g% w/v. SLIDE 11 clearly shows such a colony (0.5 cm. diameter).

Bacterial cultures were normally diffuse and non-flocculent in character particularly if spherical or discreet as in SLIDE 12 which shows a Serratia spp. Although rod-shaped varieties gave loose irregular flocs with approximate diameters up to 1.0 cm after prolonged continuous culture. SLIDE 12 shows the initial development of a bacterial floc of this type. This has enabled tower continuous acetification in vinegar manufacture using Acetobacter spp. Experimental laboratory and semi-pilot scale tower acetators have been run continuously with 80 - 90% conversion of alcohol to acetic acid at volumetric efficiencies of 1.0 day^{-1}. [2,9,10,11] However, the concentration of organism and the fermentation efficiency was entirely dependent on aeration although the fermentation shows some heterogeneity depending on the aspect of ratio of the fermenter.

Filamentous fungi usually formed pellets which were either compact or loose depending on culture conditions and reflecting the morphology of the mycelium. Various fungi were tested SLIDE 14 shows the Fungi imperfecti most of which gave compact pellets (except Fusarium oxysporum which had a yeast-type morphology). SLIDE 15 lists the best growing Basidiomycetes and Phycomycetes which gave similar morphological forms. Aspergillus niger was chosen for particular study[4,5] and under submerged conditions varied from diffuse growth through a range of pellet sizes up to several millimetres in

diameter. Isolated pellets could be grown up to some 3 cm. in diameter. In certain fermentations control over morphology is advantageous and the tower fermenter facilitates this control[5,12,13,14]. The morphology of a pellet is largely dictated by the form and size of the inoculum used to seed the fermentation, the degree of agitation a few hours after seeding the fermenter, the method of preparation of the inoculum and the growth conditions.

SLIDE 16. Filamentous colony-form.

SLIDE 17, 18, 19, 20, 21, 22. Development of soft-colonies.

SLIDE 23. Large isolated colonies up to 3 cm. diameter.

These slides illustrate the development of soft 'colonies' up to 0.5 cm. diameter and are generally obtained with spore inocula of between 10^2 to 10^6 spores per ml.; the colony size after 24 hr. reflecting the number of spores. Such inocula germinate and then aggregate to form a dense clump, this grows rapidly forming in the first stages a light 'hairy' pellet but at later stages it becomes more dense internally as well as increasing in volume appearing smoother in character. Finally, in isolated culture, such colonies lyse in the centre and become hollow with a convoluted surface.

Spore-inocular below 10^2 spores per ml. tend to give filamentous forms or may not germinate at all, while in excess of 10^6 spores per ml. tend to give dense filamentous growth. Mycelial (homogenised) inocula give filamentous growth above concentration of 0.08 to 0.10 g% w/v but may form loose aggregates whilst below this concentration smooth aggregates are formed. Where the growth conditions are controlled, for example, in the use of inhibitors to modify metabolism as in the case of citric acid production using ferrocyanide, then often a restricted growth occurs giving rise to smooth and hard pellets with hollow centres due to lysis[13, 14].

SLIDE 24 shows such pellets which develop up to 1 cm. diameter.

SLIDE 25 shows pellets cut open to reveal lysed hollow interior.

Finally it should be pointed out that combinations of organisms as occur in symbiotic cultures afforded by natural ecosystems allow unusual but predictable morphology in towers. A preliminary study of an activated sludge tower fermentation system has revealed that yeasts and bacteria are assisted in

flocculation or pellet formation by various fungi thus giving conditions which favour the tower fermenter.

SLIDES 26 and 27 show Actinomycete fungus clearly providing a 'former' for various yeasts and bacteria to form flocs in a tower fermenter.

For chemical engineering design purposes it is not possible to predict the overall essential parameters which prescribe flocculation of all micro-organisms, thus each must be considered separately. However, flocs can be considered as close-packed spheres and the effects due to diffusion on cell-growth and metabolite production estimated, whilst fluidisation-sedimentation behaviour predicted in semi-quantitative terms related to superficial-velocity and floc-concentration[5]. Growth rate equations similar in mathematical form to the Michaélis-Menten equation are not applicable to pellet formation particularly in the case of filamentous forms. However, sedimentation phenomenon appears to resemble 2nd. order kinetics[5, 15] whilst the fermentation race processes are probably zero-order kinetics[5, 7].

Comparisons between stirred tank reactors and tower fermenters have shown that the tower system is a more efficient aerator when operating at 1 volume per volume per minute and since the power input of the stirred tank is an order of magnitude greater[16, 17, 18], the high shear rates obtained prevent aggregate formation of the pellet-type and result in the more familiar filamentous form. The lower 'viscosities' encountered with pellet forms may well improve mass transfer characteristics. There are almost no significant differences in growth rate and substrate utilisation efficiency between the two fermentation systems but the key rate-processes are only affected at high cell concentrations. Calculations show that the control over morphological form allowed by the tower fermentation system provides flexibility in determining growth rate, biomass concentration, mass-transfer of oxygen and metabolites.

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The Growth and Morphology of Microorganisms in Tower Fermenters

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SLIDE INFORMATION

SLIDE 1: Basic design of an aerobic and an anaerobic tower fermenter.
Taken from original Patents. Royston Brit. Patent:929,315.
Greenshields. Brit. Patent: 1,263,059.
Differences in aeration means, separation devices and foam control.

SLIDE 2: Commercial Tower fermenter for beer production. 10 metres x 1 metre.
Diagram 2(a) Photograph 2 (b)

SLIDE 3. Non-flocculent yeast. Magn. x 900. Saccharomyces cerevisiae showing only light aggregation.

SLIDE 4. Development of physically-limited type II. Saccharomyces cerevisiae colony.

SLIDE 5. Large colony of Type II. S. cerevisiae.

SLIDE 6. Type II x 900 showing packing.

SLIDE 7. TYPE II yeast light flocs 0.1 to 0.3 cm. diameter.

SLIDE 8. Type III yeast. S. cerevisiae fermentation-limited showing dense packing 'hexagonal' benzene ring yeast cells.

SLIDE 9. Type III. sticky flocs 1.3 cm. diameter in 'plug' form.

SLIDE 10. Type II and III. Saccharomyces carlsbergensis in tower fermenter showing floc.type.

SLIDE 11. S. cerevisiae colony-form in aerobic tower. Erythrocyte-shaped colony 1.0 cm. diameter.

SLIDE 12. A serratia spp. - not flocculent.

SLIDE 13. Activated sludge bacteria forming small compact pellet-type colonies.

SLIDE 14. Table of Fungi imperfecti cultured in tower fermenters.

SLIDE 15. Table of Basidiomycetes and Phycomycetes which grew best in tower fermenters.

SLIDE 16. Aspergillus niger 72/4 Filamentous colony form.

SLIDES 17. 18. 19. 20. 21. 22. Development of A. niger M.1. soft colony form up to 0.5 cm. diameter.

SLIDE 23. A. niger M1. Large isolated colony with hollow centre. 3 cm. diameter.

SLIDE 24. A. niger 72/4. hard, smooth citric-acid producing pellets in ferrocyanide--molasses medium.

SLIDE 25. Pellets as in Slide 24 cut open to show hollow centres.

SLIDE 26. Actinomycete fungus providing a'pellet-former' for yeasts and bacteria in activated sludge tower.

SLIDE 27. Clearer detail of SLIDE 26.