ABSTRACT

DIMORPHISM AND PATHOGENICITY OF *MUCOR ALTERNANS* (VAN TIEGHEM)

A filamentous fungus, *Mucor alternans* (Van Tieghem), was found to grow in the yeast-like form in yeast extract-peptone-glucose broth (pH 4.5 and 6.8, at 25 and 37°) under carbon dioxide, and in tissue culture medium 199 (pH 7.2, 37°) under 5% carbon dioxide, 95% nitrogen gas mixture. The amount of growth was proportional to the glucose concentration. The pathogenicity of the yeast-like cells was studied in diabetic mice (strain C57 BL/KsJ-db/db) injected intraperitoneally with pre-incubated sporangiospores. A non-fatal disease developed from which the mice recovered. Cultures of *M. alternans* were obtained from visceral organs, lungs, brain and nodules of 37.7% of the diabetic mice. Normal controls were negative in culture. Upon microscopic examination of livers and nodules, hyphae, arthrospores and budding cells were found. It is postulated that single cells, e.g., broken off arthrospores disseminate the disease to distant foci where germination occurs accounting for the presence of hyphae observed in histopathological sections.

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by

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I. INTRODUCTION AND PURPOSE

The ability of a fungus to invade human and animal tissues is related in part to its potential for a morphological transformation or adaptation to parasitism. The extent of this transformation divides most fungal pathogens into dermatophytes causing the superficial infections and systemic pathogens occurring in deep-seated organs. The dermatophytes which parasitize the keratinized layers of skin, and its appendages, exhibit "reduced morphology", a term used in mycological nomenclature to describe invasion of skin, hair and nails by means of hyphae which, in most cases, break-up into arthrospores by fragmentation of the invasive mycelium. In-vitro, arthrospores do not occur but conidia are produced in most species which aid in the identification of the causative agents.

Many pathogenic fungi causing systemic infections exhibit an extreme form of dimorphism, mostly yeast-like. In studying the factors affecting dimorphism in these fungi, it was found that in the case of Blastomyces dermatitidis and B. brasiliensis temperature is of greatest importance in inducing the yeast-like growth. Sporotrichum schenckii and, particularly, Histoplasma capsulatum, are more fastidious in this respect in that, besides temperature, nutrition, humidity and the oxidation-reduction potential play a role in the initiation of the yeast-like growth in-vitro (Romano,

1967), Coccidioides immitis requires specifically carbon dioxide for the conversion to its spherule stage in a complex synthetic medium (Lones and Peacock, 1960).

The opportunistic pathogenic yeast Candida albicans does not appear to belong to the above group since both filaments and yeast forms are frequently found in lesions. Winsten and Murray (1956), claimed they found a filamentous mutant of C. albicans which was non-pathogenic for mice upon intravenous inoculation, but that culturing on cysteine containing media, restored both pathogenicity and the yeast-like growth in-vitro. A similar response to cysteine was observed by Rippon and Scherr (1959), who succeeded in obtaining yeast-like growth in Trichophyton rubrum and Microsporum audouini. In 1965, Rippon et al converted even species of Aspergillus and Penicillium to yeast-like growth on cysteine-containing media. In both cases, the unicellular growth subsequently invaded internal organs which led these authors to conclude that given specific conditions, dermatophytes and some saprophytes can become systemic pathogens.

Several members of the Lower Fungi belonging to the Class Zygomycetes, specifically Rhizopus, Mucor and Absidia can, under opportunistic conditions, become pathogenic for man. Since histopathological sections often prepared post-mortem show the characteristic broad non-septate hyphae in internal organs these fungi are

considered to be invasive in their filamentous form, i.e., in the form they grow saprophytically in nature (Straatsma et al. 1962). Some Zygomycetes, especially Mucor, have long been known to grow in the yeast phase in-vitro under specific conditions. Bartnicki-Garcia, Nickerson and others, whose work will be discussed in details in the Review of Literature, determined the requirements for pCO_2 and the kind of hexose as well as its concentration, for the conversion of these fungi to budding forms. Let is suffice at this point that the conversion to the yeast phase in, e.g. Mucor rouxii is a relatively simple laboratory exercise, compared to that of such diphasic pathogens as Histoplasma, Sporotrichum or Coccidioides. The authors who studied dimorphism in Mucor and other Zygomycetes, did not extend their investigation to pathogenicity, yet a possibility of dimorphism playing a part in diseases caused by them is an intriguing one. Would certain metabolic disorders, like diabetes, favour a conversion to yeast phase in the patient and thus account for a rapid spread of the infection ? Such a supposition appears to be supported by the ease with which these fungi respond to environment with morphological changes in-vitro and their fast rate of growth in either phase, or intermediate forms. This challenging hypothesis became the main subject of the investigation presented in this thesis.

Mucor alternans was chosen for this study because this species was isolated under apparently pathological conditions from

swabbed wounds of a patient, with multiple cuts and contusions (Royal Victoria Hospital, Montreal, 1962, Mycological Laboratory, Nos. 1463, 1465 and 1522). In two pilot experiments, this species was found to produce budding yeast-like cells in deep cultures, and upon injection of these cells into diabetic mice, the fungus was re-isolated 2 - 3 weeks later from internal organs (M. Leyritz and H. Adamonis, 1971/72. Unpublished data. A research course and a summer project in this Department). A Review of Literature revealed that except for an early description of this species by Gayon and Dubourg (1887), M. alternans has not been investigated for its potential dimorphism.

The in-vitro studies were performed first in the same medium (yeast extract - peptone - glucose broth), and using basically the same procedure as employed by the chief investigators of this phenomenon, Bartnicki-Garcia and Nickerson (1962 b), with M.subtilissimus, chosen for comparative studies because it is known to grow exclusively in the yeast-like form under carbon dioxide and nitrogen. The in-vitro studies were then transferred to a defined tissue culture medium-199, because of its ability to support the growth of animal cells. Since mucormycosis (syn. phycomycosis), in e.g., diabetic patients is associated with ketoacidosis, glucose, acetoacitic acid and β - hydroxybutyric acid, as well as urea, were added to this medium to see whether these compounds had any influence on the morphogenesis of this fungus.

The in-vivo studies of the pathogenicity of M. alternans were carried out in diabetic mice strain C57 BL/KsJ-db/db. These mice were injected intraperitoneally with pre-incubated sporangiospores. While an intranasal or respiratory portal of entry would have required the usage of dry sporangiospores as an inoculum, the intraperitoneal route, being comparable to stepping into a diseased state, called for an inoculum of cells already adapted to pathological conditions. According to the hypothesis presented above, pre-incubated spores constituted such a inoculum. The course of the disease was followed by culturing the organs, blood and urine during a two-month period after inoculation. Quantitative estimation of fungal elements was carried out by cultures and microscopic examination of homogenized livers. To investigate a possible post-mortem growth of the fungus, the livers of some mice were incubated at dropping temperatures from 37 to 4°C over a 48 hour period, prior to homogenization.

To summarize: the object of this study was to find answers to the following four questions:

1.- Since early observations, as well as preliminary studies, showed M. alternans to be potentially a diphasic fungus, what are the specific conditions required for the yeast-like growth of this fungus ?

2.- Is M. alternans, isolated under seemingly pathogenic conditions, really pathogenic ? Since mice of strain

C57 BL/KsJ-db eventually die of diabetes at 4 - 6 months, predisposing factors would be provided to bring out pathogenic attributes in this species, while normal mice should remain unaffected.

3.- If the answer to Question 2 is yes, how does the infection spread in the experimental animal ? Would the reduced oxygen tension and raised carbon dioxide content allow the yeast phase to develop wholly or partially, thus providing a vehicle for dissemination via single cells ?

4.- If the fungus disseminates via single cells, do the hyphae observed in histopathological sections result perhaps from a post-mortem growth whenever such tissues or organs are exposed to aerobiosis prior to fixation ?

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II. REVIEW OF LITERATURE

Dimorphism in Mucor

Mold-yeast dimorphism of Mucor has been studied for over a century. Bail in 1857 was the first to observe spherical budding cells in sugar-rich "saccharine" cultures of Mucor, and thought that the budding cells were ordinary brewer's yeasts. Bail concluded that the filamentous growth and the yeasts were two growth forms of the same fungus, i.e., Mucor. While his observations were correct, Bail's conclusions were wrong, and this is why Pasteur (1876), having observed this phenomenon himself, rejected Bail's hypothesis of transmutation of species. Pasteur argued that the morphology of Mucor became yeast-like as a result of oxygen deprivation. When the fungus was grown in liquid cultures, filamentous growth was obtained at the surface of the medium, where the supply of air was plentiful, while yeast-like growth developed in the depth of the same culture where aeration was insufficient. Reess (1870) and Fitz (1873), had previously reached the same conclusion. Pasteur studied in this respect several fungi and noted that the tendency to form yeast-like cells was much stronger in Mucor than in Aspergillus or Penicillium. Gayon and Dubourg gave in 1887 a good description of growth forms encountered in Mucor alternans (Fig. 1 to 10).

> "Ensemensé dans de l'eau de levure, par exemple, il produit un mycelium unicellulaire, très ramifié, qui

donne naissance en différents points à des filaments fructifères, dont les principales formes sont dessinées dans les figures 6, 7 et 8 de la planche..."

"Les spores du Mucor alternans sont elliptiques, à surface lisse, et mesurent de cinq à six millièmes de millimètre de longueur sur deux à trois millièmes de millimètre de largeur (Fig. 5); elles sont renfermées dans une membrane incrustée de petites aiguilles cristallines, et groupées autour d'une columelle sphérique (Fig. 4), dont la base conserve toujours, en forme de collerette, un débris de la membrane extérieure"..

"Cultivé dans une dissolution de sucre de canne, le Mucor alternans se développe exclusivement en mycélium et fructifie comme dans l'eau de levure non sucrée".

"Au contraire, dans une dissolution de glucose, il prend immédiatement l'état de grosses cellules sphériques très bourgeonnées (Fig. 10), et provoque une fermentation active. Dans du moût de bière, dans des dissolutions de maltose, de dextrine, et même de glucose impur, dans de l'empois d'amidon, il produit d'abord, des tubes mycéliens qui se gonflent bientôt, se cloisonnent et forment une succession d'articles à peu près cylindriques (Fig. 9 a)

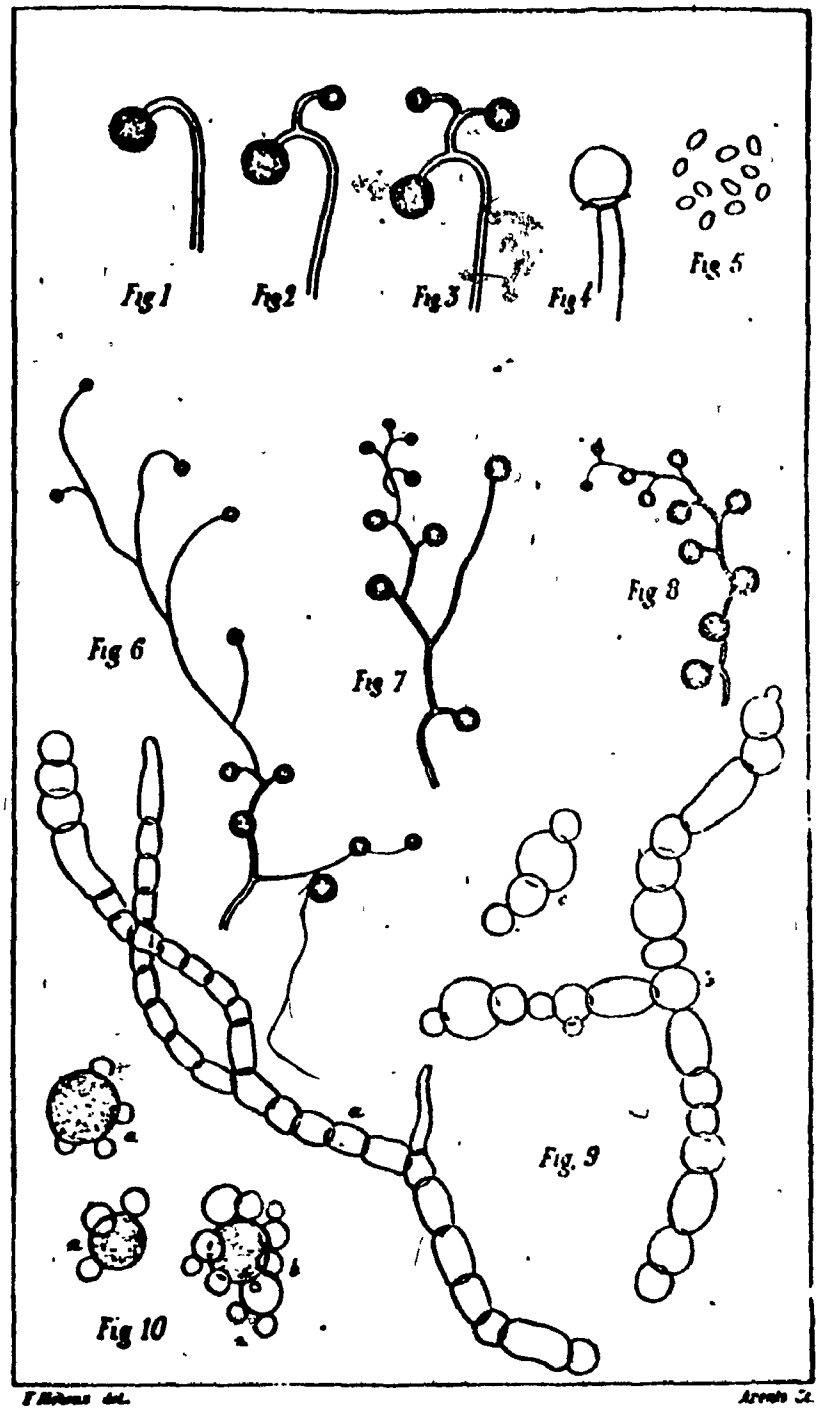


Fig 1
Fig 2
Fig 3
Fig 4
Fig 5
Fig 6
Fig 7
Fig 8
Fig. 9
Fig 10
a
b
c

EXPLICATION DE LA PLANCHE

Fig. 1. — Filament fructifère du *Mucor alternans* recourbé en crosse et terminé par un sporange sphérique. G = $\frac{200}{1}$.

Fig. 2. — Filament fructifère avec deux sporanges alternes. G = $\frac{200}{1}$.

Fig. 3. — Filament fructifère avec trois sporanges alternes. G = $\frac{200}{1}$.

Fig. 4. — Columelle sphérique lisse, portant à sa base un reste de la membrane extérieure du sporange. G = $\frac{500}{1}$.

Fig. 5. — Spores elliptiques du *Mucor alternans*. G. = $\frac{500}{1}$.

Fig. 6, 7 et 8. — Aspects divers des organes de reproduction du Mucor. G. = $\frac{100}{1}$.

Fig. 9. — États successifs du mycélium dans une solution de dextrine : *a*, filament né d'une cellule ferment, divisé en articles cylindriques ; *b*, filament dont les articles sont gonflés et arrondis ; *c*, articles détachés du filament précédent. G = $\frac{500}{1}$.

Fig. 10. — Cellules-ferment en voie de multiplication dans un liquide en fermentation : *a a*, cellules peu bourgeonnées ; *b*, cellule-mère entourée de cellules nombreuses, de première et de deuxième génération. G = $\frac{300}{1}$.

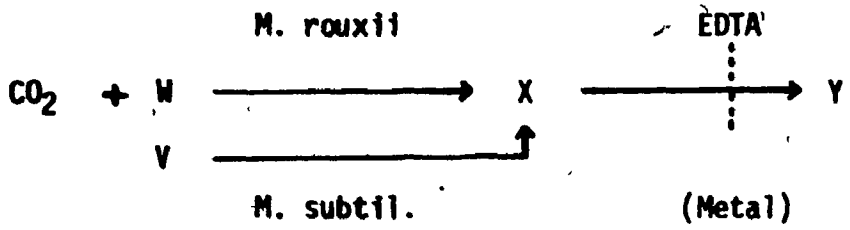
puis, ces articles s'arrondissent en boules (b et c), se séparent les uns des autres et se reproduisent finalement à l'état de cellules sphériques, pendant toute la durée de la fermentation alcoolique."

About the same time, Brefeld (1873) observed M. racemosus to grow in the filamentous form in anaerobiosis under hydrogen. He concluded that the formation of yeast-like cells was due to the accumulation of metabolic CO_2 and not to the absence of oxygen, the acidic properties of CO_2 being responsible for this effect. Klebs (1896) having doubts about the role of acidity in yeast-like development, showed that he could obtain yeast-like cells in neutralized grape juice, provided the air was completely excluded. Whemer (1905) also found that the absence of air was the most important condition for the appearance of yeast-like development of M. guilliermondii under anaerobic conditions by incubation under CO_2. In these early studies, it became gradually evident that the atmosphere of incubation, i.e., absence of air, and presence of CO_2 played an important role in determining the morphogenesis of Mucor. The mechanism of these changes, however, was little understood.

The modern phase in research of dimorphism in Zygomycetes concentrating on Mucor, began when Bartnicki-Garcia and Nickerson undertook, in 1962, critical and extensive studies of the physiological control of dimorphism in Mucor. The importance of distinguishing

between arthrospores and yeast-like cells was emphasized and the term "budding cells" clarified. According to these authors (1962 b), the term "yeast-like cells" should apply only to cells which originate and multiply by budding and not to arthrospores, which arise by hyphal fragmentation. Much of the confusion in earlier work arose from the failure to distinguish between these two forms of cells. Carbon dioxide and nitrogen were confirmed to control dimorphism in the several species of Mucor studied, although not all species behaved similarly. M. rouxii was notable for its yeast-like growth only under carbon dioxide while M. subtilissimus produced this type of growth under both carbon dioxide and nitrogen. Under anaerobic gas mixtures of carbon dioxide and nitrogen, a pCO_2 of 0.3 atmosphere induced the development of exclusively yeast-like cells in M. rouxii while under aerobiosis a pCO_2 as high as 0.9 atmosphere was required to obtain a predominantly yeast-like growth (1962 b). Examination of the action of metabolic inhibitors on M. rouxii (1962 c), led to the discovery that morphogenesis was susceptible to control by chelating agents of the N-acetic acid type, such as EDTA (ethylenediaminetetraacetic acid). Yeast-like development in M. rouxii was prevented by EDTA, and restored by subsequent addition of such metals as Mg, Zn and Mn, showing that morphogenesis is metal-dependent. Since yeast-like development in M. subtilissimus was also inhibited by EDTA, Bartnicki-García postulated that yeast-like development in these two species is dependent upon the metal-catalized

formation of an important metabolite. In order to form this metabolite, CO_2 would be required in the case of M. rouxii, but not in the case of M. subtilissimus. The following model was proposed:



where V, W and X are metabolic precursors of Y, the action of Y affecting the chemical composition of the cell wall, and consequently the cell shape.

It was also found that absence of oxygen greatly reduced the amount of growth and increased the nutritional demands of the fungus. Thiamine and nicotinic acid were required for anaerobic growth of both the yeast-like form and the filamentous form of M. rouxii, while no added vitamins were necessary for aerobic growth (Bartnicki-Garcia, S., and W.J. Nickerson, 1961). The same authors found nutrition to be of paramount importance. Abundant yeast-like development was obtained only with complex nitrogen sources such as yeast-extract-peptone or casein hydrolysate but not with single amino acids (1962 c). The importance of the medium on growth was also emphasized by Haidle and Storck (1966 b), who succeeded in obtaining yeast-like growth of M. rouxii under nitrogen in synthetic media provided traces of oxygen were eliminated.

Assimilation of carbon was also found to be affected by anaerobiosis. Under aerobic conditions, disaccharides and a variety of organic compounds were utilized, while in absence of oxygen carbon assimilation was restricted to hexoses (Bartnicki-Garcia and Nickerson, 1962 c). Flores-Carreon et al (1969), proposed a possible explanation for this phenomenon. They found that only cell-free extracts of M. rouxii could hydrolyze maltose under anaerobic conditions, while whole cells required oxygen for this process. A functional respiratory chain seems to be required for maltose entry into the cell since impairment of the electron transport by amytal inhibited maltose uptake. These results rule out the possibility that oxygen is necessary for maltose breakdown or for the inducible synthesis of α-glucosidase. These researchers are also against the hypothesis of Kluyver and Custer (1940), that certain yeasts are unable to ferment some disaccharides because the enzymes involved in their hydrolysis are reversibly inactivated under anaerobic conditions. The importance of hexoses was again noted later by Bartnicki-Garcia (1968), who proved that in anaerobic cultures of M. rouxii, morphogenesis was strongly dependent on hexose concentration, as well as pCO_2. At low levels of hexose, or pCO_2, or both, hyphal development occurred, but at high levels, the fungal spores developed into yeast-like cells. When the spores were incubated under nitrogen, at a low glucose concentration, as in the 2% glucose yeast-extract peptone broth, germination occurred

and long thin hyphae were obtained. But when glucose, in that medium, was raised to 8%,only budding cells were obtained, proving that at high glucose concentration, yeast-like development could be obtained without exogenous CO_2. Characteristically high glucose concentrations inhibited in this species the development of hyphae from germinating spores, but did not prevent the elongation of preformed hyphae, suggesting that hexoses inhibit formation of hyphae not by blocking the operation of the mechanism responsible for apical growth but by preventing its initiation.

Considerable light was thrown on dimorphism in Mucor by chemical investigation and comparison of the cell wall composition of the yeast-like and mycelial phases of M. rouxii. Both types of cell walls exhibited a complex chemical composition consisting of polysaccharides, phosphates, proteins, lipids, purines and pyrimidines, also magnesium and calcium. Chitosan was the most abundant component of both types of cell walls while chitin was present in relatively small quantities (Bartnicki-Garcia and Nickerson, 1962 a). Chitin was found later to play no role in morphogenesis (Bartnicki-Garcia and Lippman, 1972). Significant quantitative differences, however, were found: proteins, pyrimidines and particularly mannose were present in much higher quantities (5 times as much in the case of mannose), in the yeast-like cell walls than in hyphal wall (Bartnicki-Garcia and Nickerson 1962 a).

Other chemical analyses aided in the elucidation of the role of CO_2 in Mucor morphogenesis. Working with labelled carbon, Bartnicki-Garcia (1962 d), found that carbon dioxide was fixed by the yeast-like cells of M. rouxii. Grown anaerobically under 0.3 $pC^{14}O_2$, maximum fixation of the labelled carbon dioxide and maximum malic enzyme activity occurred. The major product of $C^{14}O_2$ fixation was found to be aspartic acid. The two above findings, i.e., the high mannose content and the fixation of CO_2 in aspartic acid, led Bartnicki-Garcia to the following hypothesis: since CO_2 assimilation at high pCO_2 resulted in large accumulation of malic acid, which is then metabolized and converted to aspartic acid, the latter compound, i.e., aspartic acid, would stimulate the formation and accumulation of mannan protein in the cell wall. The increased presence of the mannan protein would disrupt, or prevent, an orderly orientation of the cell wall polymers and through the action of isotropic forces the cell would become non-polarized, i.e., spherical. This hypothesis supports earlier work of Kessler (1959), who found that the protein moiety of the mannan protein complexes isolated from yeast cell walls is also rich in aspartic acid. By contrast, under aerobic conditions, no accumulation of carboxylic acids would occur as a result of CO_2 fixation, since they would be removed through the operation of the tricarboxylic acid cycle.

Recently, other factors besides the atmosphere of incubation, glucose concentration and nutritional factors, were found to induce dimorphism in Mucor. Phenethyl alcohol (PEA) is one of them. This alcohol is known to inhibit the growth of bacterial fungal and animal cells. Previous studies on the mode of action of PEA suggest that the cytoplasmic membrane is its primary site of action (Silver et al. 1966). The germination of fungal spores of M. rouxii was inhibited by concentrations of phenethyl alcohol ranging from 0.05 to 0.3%. Under aerobiosis in the presence of 0.22% PEA, M. rouxii spores gave rise to an abundant amount of budding spherical cells, provided that the carbon source was a hexose at 2 to 5%. Removal of these cells to PEA free medium resulted in germination, and hyphal growth (Terenzi and Storck, 1969). Yeast-like development was also obtained in a basidiomycete (Lenzites saepiaria) upon treatment of its basidiospores or mycelium with PEA (Scheld et al, 1970). PEA did not inhibit synthesis of cytochromes or O_2 uptake, but was found to inhibit the oxidative-phosphorylation activity of mitochodria according to Terenzi and Storck (1969). The net result of the action of PEA was stimulation of CO_2, and alcohol production. The above results suggest that filamentous growth inhibition by PEA could be the result of an uncoupling of oxidative phosphorylation from respiration and that in Mucor, yeast-like morphology and fermentation are linked. Yeast-like morphology even in aerobiosis would be expressed only when alcoholic fermentation reaches a critical level. Yeast-like morphology

in Mucor can thus be observed to occur under diverse conditions, involving CO_2, hexose concentration or PEA. These conditions have one common trait: they all favour fermentation over respiration.

In 1971, Storck and Morill reported the isolation of a mutant of M. bacilliformis, which had lost the ability to grow in the filamentous form and grew exclusively as yeast-like cells. The inability of the mutant to take up O_2 in the presence or absence of glucose, was found to be due to the lack of cytochrome-oxidase. This mutant was unable to take up O_2, but had a high level of alcoholic fermentation. Fermentation in this case was its sole source of energy. The discovery of a cytochrome-oxidase negative mutant was paralleled by the findings of Haidle and Storck (1966 b), who studied the enzymatic reactions involved in the conversion of yeast-like cells to filaments in M. rouxii. On exposure of the yeast-like cells to air, they found that cytochrome oxidase was necessary for the conversion to filaments to occur. Addition of cyanide, acryflavine and cycloheximide which inhibit the activity or synthesis of cytochrome oxidase also prevented the conversion. On the other hand phenethyl alcohol which inhibits the filamentous but not the yeast-like growth, had no effect on the activity or synthesis of this enzyme. It was then concluded that besides cytochrome oxidase, other enzyme systems which are inhibited by phenethyl alcohol are required for the conversion to occur. Respiration which requires the presence of cytochrome oxidase

and other enzyme systems would then favour filamentous growth, while fermentation whether induced or not would favour yeast-like growth.

Considering that the yeast phase of M. rouxii is accompanied by a high level of fermentation, it seems likely that the enzymes of the glycolytic pathway may participate in the control of morphological change. Crude extracts of yeast-like cells, exhibited 3 to 5 times more total specific activity of pyruvate kinase than those from the mycelium. Manganese ions were found to be strong activators of this enzyme (Passeron et al. 1970). This finding is consistant with the fact that in anaerobiosis the catabolism of glucose through glycolysis requires the formation of pyruvate from phosphoenol pyruvate while in aerobiosis pyruvate kinase would not play as an important a role in energy production.

It was also found that spores of several species of Mucor are converted into the yeast-like form under aerobic conditions in presence of suitable concentrations of certain antibiotics and fungicides. At 10^{-5}M., actinomycin D was the most effective antibiotic producing yeast-like forms in M. hiemalis, M. pusillus, M. rouxii and Actinomucor elegans. The fungicide thiabenazole produced yeast-like cells in all these species at 5×10^{-4}M, griseofulvin, streptomycin, tyrocidine, dicloran, ethirimol, dimethirimol, carboxin and oxycarboxin induced yeast-like growth at 10^{-4}M (Fisher et al., 1973).

Haidle and Storck (1966 b), found that in the presence of cycloheximide, the morphological conversion of yeast-like cells to filamentous cells, was inhibited. Since it is known that protein and RNA accompany this conversion (Haidle and Storck, 1966 a), and that cycloheximide inhibits both of these syntheses, it was assumed that protein and/or RNA synthesis, were necessary for the yeast to filament change to occur, and that this change involved more than merely the change in shape of the cell wall.

In summary, fermentation and environmental conditions, which enhance fermentation, restrict morphology in Mucor giving rise to yeast-like cells. This concept is in agreement with the views of Warburg (1968), regarding oncogenesis: "Respiration energy creates and maintains a high differentiation of body cells. Fermentation energy can only maintain a low differentiation. It follows that if respiration is replaced by fermentation in body cells, high differentiation must disappear."

Phycomycosis in man and in experimental animals (Syn. Mucormycosis).

Phycomycosis is a systemic disease in man and animals, caused by several members of the orders Mucorales and Entomophthorales, including such genera as Mucor, Absidia, Rhizopus, Entomophtora and Basidiobolus. This is an opportunistic infection in patients debilitated by another disease and receiving complex therapy. The first report of phycomycosis

in man, was made by Paltauf (1885). As early as 1903, it was confirmed by Barthelet that Mucor was an invader of tissues previously altered by disease and was rarely a primary pathogen. In 1943, Gregory et al. reported three cases of cerebral mucormycosis in patients with diabetic acidosis. McBride et al (1960), noted that 42% of the cases reported in their review, occurred in patients with diabetes, most of whom had been severely acidotic. In non-diabetic patients, the most predisposing conditions to invasion by Mucor would be: 1) a state of lowered resistance produced by underlying diseases, such as carcinoma, anemia, glomerulonephritis, septicemia, leukemia, multiple myelloma and metabolic disturbances, other than diabetes, leading to acidosis; 2) a local tissue defect that the fungus uses as a portal of entry, as in the case of a burn wound; and 3) a disturbance brought about by the use of antibiotic, cytotoxic, steroid and radiation therapy.

It has been noted by McBride et al, in 1960, and Straatsma et al, in 1962, as well as by other workers (Abramson 1967, Becker, 1968, Bergstrom 1970, Blatrix 1970, Burrow 1963, vonLedebur 1968), that cerebral phycomycosis was prelavent in acidotic diabetic patients. The fungus is thought to spread by direct extention from the nasal sinuses as portal of entry to the brain. The disseminated form has occurred most frequently in non-diabetic non-lymphoma patients, while the pulmonary form seems to affect mostly lymphoma patients (Straatsma

1962). Two other forms; gastro-intestinal (Deal et al. 1969), and subcutaneous phycomycosis occurred in a few instances (Straatsma 1962).

Phycomycosis is rarely suspected clinically and, since mycological diagnosis is either not attempted or negative, except in rare instances, (Bauer et al 1955 a), most cases proceed to a fatal issue, and are diagnosed post mortem by pathologists, by demonstration of the characteristic broad, non-septate invasive hyphae and tissue reactions. Unfortunately, the pathologist cannot go any further and thus phycomycosis is notoriously ill-defined as to causative species. Even when cultures are obtained, ante- or post-mortem, identification of the genera, not mentioning the species, is a matter for experienced mycologists familiar with that group of fungi. It is because the course of this disease is largely unknown that studies of its pathogenesis have been undertaken in animals by several investigators.

Lichtheim (1884), in the first experimental approach to the study of phycomycosis, gave intravenous injection of M. corymbifer and M. rhizopodiformis to rabbits producing widespread lesions and death. Since cerebral mucormycosis appears chiefly in patients with diabetic acidosis, Bauer et al (1955 b), studied mucormycosis in rabbits made diabetic by injecting alloxan into their blood stream. They found that intranasal inoculation of Rhizopus spores produced extensive nasal, pulmonary, cerebral and kidney lesions which closely

resembled those in man. Only minute lesions at the site of inoculation were observed in normal rabbits. They concluded that metabolic disturbances associated with diabetes mellitus would be an essential factor in pathogenesis of phycomycosis. Similar results have been obtained by Schofield and Baker (1956) with mice. Elder and Baker (1956) had proposed that hyperglycemia and/or acidosis, and shock, may account for the pronounced hyphal growth in acutely toxic diabetic rabbits.

In order to see whether hyperglycemia is indeed a predisposing factor in diabetes, sustained hyperglycemia in rabbits without diabetes was studied (Bauer et al 1956). It was found that rabbits inoculated with Rhizopus oryzae spores developed pulmonary lesions which,although more numerous and extensive than in normal animals, did not approach the severity of the lesions in rabbits with acute alloxan diabetes. They concluded that hyperglycemia would not be the sole predisposing factor, that it would be an intermediate stage in host susceptibility. Other factors like ketones and the activity of polymorphonuclear leukocytes would then play a role in pathogenesis. Baker et al (1956) as well as Schofield and Baker (1956), also found that hyperglycemia by itself did not increase the host susceptibility to mucormycosis. On the other hand, Drachman et al (1966) working with bacteria, found no impairment of the inflammatory response in the non-diabetic ketotic rats, but a failure of the localized leukocytes to ingest the invading organism.

In their study of anti-bacterial defence, they found that glucose was the factor in serum which depressed phagocytosis. It was not verified if the same could apply to fungi.

Ketones were found to have considerable importance in the disease process. Bauer and Sheldon (1958) could obtain the production of subcutaneous granulomata by injecting spores of R. oryzae into normal rabbits. In these rabbits no fungus proliferation occurred and the lesions healed. On the other hand, when rabbits with such granulomata were subsequently rendered diabetic by alloxan, reactivation of infection was observed only in accutely acidotic diabetic animals. The skin lesions in these animals showed proliferation of the fungus and they differed from the controls by the presence of budding spores and the formation of mycelia. It was clear in that experiment that activation of the infection occurred only in the presence of acidosis. Acidosis was also found by Cruickshank (1954) to be important and he proposed that ketosis might interfere with metabolic processes in tissues and predispose to mucormycosis.

Sheldon and Bauer (1959), showed that the increased susceptibility of the diabetic and acidotic host to experimental mucormycosis was in part related to a deficiency of the early body defences during the first few hours after inoculation. A study of the morphology of early lesions in normal and diabetic rabbits, revealed that the response by polymorphonuclear leukocytes in the abnormal

host is markedly delayed, less intense and less effective and fibroblastic proliferation is lacking. These findings would indicate that if an abnormal host is exposed to the infectious agent, it will allow proliferation of the agent and extensive damage can be done before the host defence becomes active. The study of the role of the inflammatory response was then pursued.

In a subsequent experiment, Sheldon and Bauer (1960), studied a relationship between the discharged tissue mast cell granules and eosinophilic granulocytes in rats. In normal rats, discharge of tissue mast cell granules occurred within minutes at the site of infection, and appeared to initiate a rapid onset of acute inflammation. The degranulation of tissue mast cells subsided in a short time and inflammation led to the formation of localized lesions. In the rats, treated with 48/80* which causes degranulation of the tissue mast cells, the onset of inflammation was briefly delayed, the fungus growth in form of budding spores and mycelia in early lesions was increased but the infection did not spread and the lesions remained localized. On the other hand, in diabetic acidotic rats, tissue mast cells failed to discharge their granules and the inflammatory response was delayed and decreased, and the infection progressed, rapidly invading adjacent tissues. It was concluded that a function of the tissue mast cells in the normal rat was the rapid initiation of acute inflammation at the site of injury and that degranulation of these cells prior to infection

* Compound 48/80, a histamine-liberator: a condensation product of p-methoxyphenethylamine with formaldehyde.

using 48/80, delayed the inflammatory response and as a result diminished host resistance. A severe metabolic disorder such as alloxan diabetes with acidosis would then inhibit the normal function of tissue mast cells, decreasing the inflammation and increasing the susceptibility of the host to infection.

Other factors were found to influence the fate of the organism in the body. Lehrer (1970) found that serum was necessary for optimal phagocytosis of Aspergillus fumigatus spores by human leukocytes. The viability of the spores was not affected by serum even after phagocytosis by normal neutrophils and monocytes. Gale's report (1961), maintains that a non-specific inhibitor of Rhizopus spores is present in normal serum.

The effect of the depression of the inflammatory response by cortisone was also studied. Baker and Schofield (1956), found that experimental peritoneal R. oryzae infection in cortisone-treated mice, rats and guinae pigs, spreads to extraperitoneal organs to a much greater extent than in normal controls. More hyphal forms of the fungus were noted in the lesions of hormone-treated animals. It was concluded that cortisone has a tendency to promote spreading of the fungus in the animal body. Bauer, Wallace and Sheldon (1957), on the other hand, found that cortisone in the amount given in their study did not produce conditions suitable for the development of phycomycosis with unrestrained proliferation of R. oryzae in the

tissues. The hormone, however, altered the host resistance to the fungus, as shown by the increased incidence of lesions, which were about three times more common in hormone-treated rabbits than in normal controls. Furthermore, budding spores, hyphae and mycelia occurred regularly in nasal lesions of cortisone-treated rabbits, but were not generally found in the lesions of normal controls. Despite this enhanced proliferation of the fungus, the lesions in the cortisone-treated rabbits resembled those of metabolically normal controls, and did not approach the extent of severity of the fungus lesions in rabbits either with hyperglycemia or with acute alloxan diabetes.

Virtual absence of fungus growth in the inflammatory lesions, produced by croton-oil (Bauer and Sheldon, 1957), indicated that a pre-existing focus of inflammation in a metabolically normal host does not favour the development of mucormycosis. The inability of the fungus to invade tissues was thought to be related to the presence of large numbers of PMN leukocytes, which appeared normal, while in contrast the PMN leukocytes in acute alloxan diabetes or infusion hyperglycemia are also present in large numbers, but show marked degenerative changes. These findings emphasize the important role of PMN leukocytes in the pathogenesis of R. oryzae infection. An adequate response of normal PMN leukocytes inhibits the development of mucormycosis, while the metabolic alterations associated with

cortisone administration, although insufficient to induce widespread infection, clearly enhances the host' susceptibility to this fungus.

After investigating mucormycosis in acidotic diabetic and cortisone treated animals, experimental mycotic ulceration was studied by Smith (1968). He found that guinae pigs receiving aspirin, which causes ulceration of the stomach, developed infection with invasion of the lymphatics by hyphae of Absidia ramosa. He postulated that damage to the mucosal membrane in this case was necessary for the establishment of the infection, and that mycotic ulcers provided a primary lesion from which fungal elements were disseminated to other organs. This author recently found (1973) an affinity of Absidia ramosa injected intravenously into mice, for kidney and brain tissues. The possible explanation for this tissue predilection would be that renal and central nervous system lesions are due to the germination of a few fungal spores which have become trapped in regions reasonably free of phagocytic cells.

In conclusion, it appears that conditions like diabetic acidosis, cortisone treatment and immunological impairment would predispose the debilitated patients to mucormycosis, because they would adversely affect the inflammatory response allowing proliferation of the fungus in the host. It is important to note that in the majority of the experiments, the normal controls do not develop the

disease proving that phycomycosis is indeed an opportunistic infection. Josefiak (1958), injected R. oryzae spores in Selye's granuloma pouch of healthy rats. The fungus excited a non-specific inflammation reaction which became granulomatous in type with the formation of grey yellow nodules, but there was no dissemination and the animals overcame the infection. This seems to be the case for all the normal animals studied.

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III - MATERIALS AND METHODS

1.- Species Investigated

The fungus investigated in this study was Mucor alternans Van Tieghem which was isolated from torn tissues of an accident victim treated at the Royal Victoria Hospital, Montreal, in 1962. The isolated strain was subsequently identified by Dr. C.W. Hesseltine, Northern Utilization Research and Development Division, Peoria, Ill. M. subtilissimus NRRL 1909, obtained from Dr. Hesseltine, was employed for the in-vitro comparative studies.

Mucor alternans grown on Sabouraud's glucose agar, resembles other Mucor species. The long non-septate hyphae measure 10.2 μ to 15.7 μ in width. The mycelium gives rise to numerous sporangiophores which bear spore-filled sporangia. The spores are elliptical in shape and their size is about 4.6 μ by 5 μ. Mucor alternans also has a strong tendency to form arthrospores.

2.- Preparation of Inoculum

In order to ensure inocula as uniform as possible throughout the experiments, stock suspensions of spores of the above fungi were prepared in the following manner: the two species were inoculated onto several slants of Sabouraud's agar, and incubated at 25°* for 7 days. The sporangiospores were then harvested in sterile physiological saline and filtered through a sterile glass wool pad to remove any

* All temperatures reported in degrees centigrade

fragments of mycelium. The suspension of spores was then mixed with two volumes of Filde's solution containing 10% glycerine and distributed in 0.5 ml. volumes into small plastic vials. Sixty-four vials altogether were prepared and stored at -80° in a Revco freezer until required.

For each experiment a fresh vial was used to inoculate Sabouraud's agar slants. After 6 to 7 days incubation, at 25°, the spores were harvested and filtered as above. The inoculum was standardized with a hemacytometer (Bright-Line American Optical), to contain 10^6 spores/ml. for the in-vitro experiments, and 10^7 spores/ml. for the in-vivo experiments.

3.- In-vitro Growth Experiments.

a) Media

Two media were used yeast extract-peptone-glucose broth (this medium will be referred to in subsequent text as YPG broth), and TC Medium 199. The YPG broth of Bartnicki-Garcia and Nickerson 1962 b), contained per liter: 3g yeast extract, 10g neopeptone, and 20g glucose, (all 3 of the Difco Laboratories), dissolved in distilled water. The pH was adjusted to either 4.5 with 1 $N_2H\ SO_4$ or 7.3 with 1 N NaOH. The media were distributed in 50 ml. volumes into 250 ml. Erlenmeyer flasks and autoclaved at 121° for 22 min. The autoclaving lowered the pH in the alkaline medium to 6.8.

The TC Medium 199 (Difco Code No. 5696-72), purchased as a ten-fold concentrate, was used alone or with one or more of the following additives; urea (Fischer Scientific Company), β-Hydroxybutyric acid, as sodium salt (Nutritional Biochemical Corporation), acetoacetic acid, as lithium salt (Sigma Chemical Company), and glucose (Difco Laboratories). Urea and β-hydroxybutyric acid were added at a final concentration of 0.05 and 0.1%, acetoacetic acid at 0.1%, and glucose to a final concentration of 0.5, 1 and 2%. The additives were weighed for each combination, dissolved together in double distilled demineralized water, and filtered under positive pressure through a membrane millipore filter (0.22 u, Pyrex microanalysis filter holders XX-1002500). 15 ml. of the sterile TC Medium concentrate were then added to 135 ml. of the particular solution of additives and the pH adjusted to 7.3 by the addition of 0.525 ml. of 10% sterile sodium bicarbonate at 37°. The medium was distributed in 50 ml volumes into 250 ml sterile Erlenmeyer flasks.

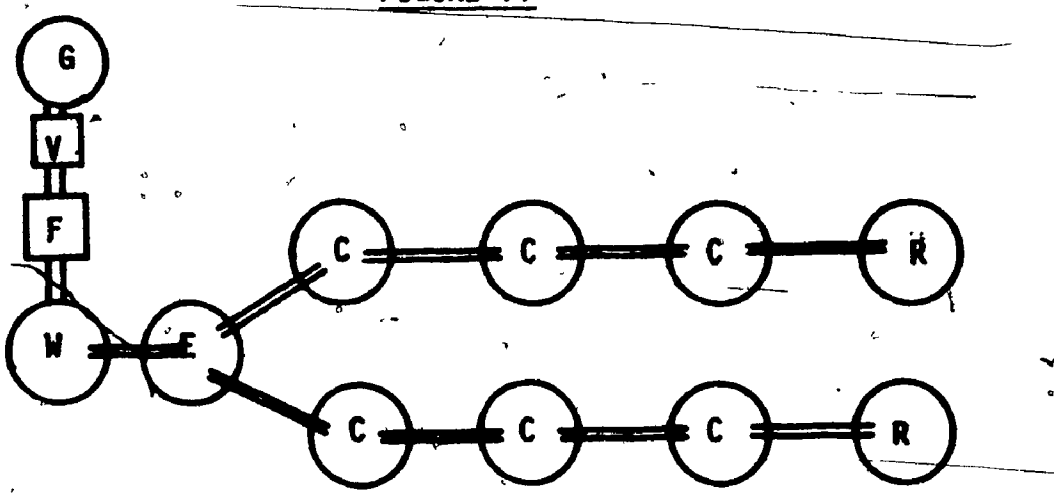
b) Apparatus and gases.

Shaking cultures.

Most of the experiments carried out under controlled gaseous conditions were incubated on two kinds of shakers (New Brunswick Scientific Co., New Jersey), producing 98 strokes per min. The flasks prepared with YPG broth were agitated on the reciprocal water bath shaker, but because this model functioned poorly at 37°, the TC Medium 199 cultures were agitated on the more sturdy gyrotory shaker. Each

experiment consisted of 6 cultures in 2 series, and 4 functional flasks connected by rubber and glass tubing (ID 4mm) to a cylinder of compressed gas. The arrangement of the flasks on the shaker was as illustrated schematically in Fig. 11.

FIGURE 11



The incoming gas (G) was passed through an extra fine needle valve (V), filtered through cotton wool filter (F), saturated with moisture by bubbling through sterile distilled water (W), passed through an empty flask (E), and distributed to triplicate culture flasks (C). The last two flasks in each series contained distilled water (R) through which the gas flow was regulated to 3 bubbles per second, a rate equivalent to 120 ml per minute.

Stationary cultures

Stationary cultures (under controlled air flow) were prepared and set up exactly like those just described, except that the

shaking mechanism was not switched on. Cylinders of compressed air, two simple gases and two gas mixtures were used in the experiments. The YPG broth experiments were carried out under air, carbon dioxide (99.5 to 99.7% pure), or nitrogen (99.95% pure), supplied by the Ohio Chemical Co., Montreal. The TC Medium 199 experiments were incubated under two gas mixtures (Liquid Air Company, Montreal), 1) oxygen 12.65%, carbon dioxide 4.99% and nitrogen 82.36% and 2) carbon dioxide 5.1% and nitrogen 94.9%. In the text the above percentages will be expressed in an abbreviated manner as follows:

Carbon dioxide 99.5 to 99.7% pure as 100%, while 4.99 and 5.1% components in mixtures as 5%.

Nitrogen 99.5 to 99.7% pure as 100%, while 82.36 and 94.9% components in mixtures as 82 and 95% respectively.

Oxygen 12.65% component in mixture as 13%.

c) Inoculation and incubation

The flasks of prepared medium (YPG broth or TC 199) were inoculated each with 1 ml of inoculum resulting in a final concentration of 2×10^4 spores per ml, and were connected with the autoclaved tubing on the shaker. In actual procedure, an apparatus composed of 10 connected dummy flasks, all strategic points carefully wrapped in aluminum foil, was autoclaved at 121° for 30 min. and placed on the

shaker. The culture flasks inoculated on the laboratory bench, and the sterile water flasks were then taken to the shaker and exchanged with the corresponding dummy flasks. (This method seems to have been good, since no contamination occurred during the whole study). The gas was then connected to the system and allowed a steady flushing during the entire time of incubation.

The YPG broth cultures were incubated at 25° and 37° while the TC Medium 199 cultures were incubated at 37°only. The incubation lasted for varying periods of time, 24, 48 or 67 hours. Incubation terminated, the growth was studied by qualitative and quantitative methods.

d) Sampling for morphological studies.

Gross appearance of the cultures was noted and 0.3 ml of a well stirred suspension was drawn from each culture flask for microscopic examination. These aliquots were stored in the cold and the examination carried out within hours, in most cases the same day.

e) Dry weight determinations

The YPG broth cultures were autoclaved at 121° for 25 min., filtered through a membrane millipore filter of 1.2 u pore size, washed several times with distilled water, dried at 85° for 24 hr. and placed in a dessicator over Drierite* until weighed. The weighing was done to constant readings on a Mettler H20 analytical balance.

(*) Drierite is anhydrous $CaSO_4$ (Hammond Drierite Co.)

The growth in the TC Medium 199 cultures, was harvested by centrifuging at 230 x G for 30 min., the pellet washed in distilled water, and dried to constant weight as above.

The portion of growth removed in the 0.3 ml withdrawn for morphological studies was ignored as insignificant (0.6%) in the determination of dry weight.

f) Residual glucose and pH determinations.

Residual glucose and pH were determined in the TC Medium 199 cultures only. After the removal of growth for dry weight determinations, the supernatant was divided into two portions. One portion was used for pH determinations which were carried out immediately, i.e., within minutes after centrifuging, with a Beckman Zeromatic pH meter, while the other was stored, frozen, and glucose determined at a later date by the Glucostat semi-micro method (Worthington Biochemical Corporation, New Jersey).

4.- In-vivo Experiments

a) Mice

100 black male mice (C57 BL/KsJ-db, Jackson Laboratory, Bar Harbor, Maine), i.e., 50 diabetic and 50 normal controls were used in the experiment. The mice were born on three different dates, within a two week period, and were from 9 to 11 weeks old at the start of the experiment. The diabetic mice were obviously plump and sluggish

and their coats were rather dull, compared to the controls. Ample food and water were supplied to them at all times. Their weight was recorded on the day of inoculation.

b) Inoculation technique.

M. alternans enlarged spores were used to inoculate 45 pairs of mice (a pair comprising one diabetic and one normal mouse). To produce the inoculum, 10^6 spores/ml (see Preparation of inoculum), were suspended in 50 ml of TC Medium 199, with its glucose concentration raised to 2%. The suspension was incubated at 37^0 on the gyrotory shaker, under a gas mixture of 5% carbon dioxide and 95% nitrogen, for about 6 hours. Under these conditions the spores swelled to about $\pm$ 15 μ in diameter; occasional budding cells (less than 0.1%) were observed upon careful microscopic examination. The spores were centrifuged at 230 x G for 30 min., washed three times in sterile saline and suspended in 50 ml of saline. 0.5 ml of the suspension (containing 5 x 10^5 spores/ml), were injected intraperitoneally into the mice, using 1 cc tuberculin syringes (DN 25g 5/8, Becton, Dickison & CO). A booster shot of spores prepared in the same manner was given 7 days later. The remaining 5 pairs of mice received a control injection of 0.5 ml of saline.

c) Anesthesia and autopsy.

Starting 3 weeks after inoculation the mice were sacrificed.

at the rate of 6 pairs per week. Because the planned schedule could not be followed exactly, the last pair was killed 12 weeks after inoculation. The diabetic mice were designated by odd numbers, and their controls by even numbers. On the day they were to be sacrificed, the mice were weighed and anesthetized with Nembutal (Abbot Co., stock sol. 60 mg/ml. diluted 1:5) at the dosage level of 0.06 mg/g of body weight (Pilgrim 1955). The anesthetized mice were kept on the bench for 45 to 50 min. to allow the level of blood glucose to stabilize. The fur was then wetted thoroughly with 5% lysol to minimize surface contamination, the peritoneum cut open, and the mouse exsanguinated by cardiac puncture with a sterile syringe containing traces of sodium heparin (1000 U.S.P. in 1 ml). Urine was also drawn whenever possible, by puncturing the bladder with a syringe. Using a separate pair of sterile scissors and forceps for each organ, liver, spleen, right kidney and adrenal, left kidney and adrenal, heart, lungs and brain were removed.

d) Culturing of organs and body fluids.

Cultures of each organ, except liver, were prepared by cutting into small pieces and inoculating each organ onto one slope of cotton-plugged meat extract-cerelose agar (*). Blood (0.3 ml)

(*) A medium, used routinely in diagnostic medical mycology, containing per liter: neopeptone (Difco) 10 g, beef extract (Oxoid) 3g, cerelose 10g, NaCl 5g, Bacto-agar (Difco)20 g, and streptomycin 1 g.

and urine (all but a few drops), were also cultured on that medium. The cultures were incubated at 25° and were examined for growth twice weekly for 3 weeks.

e) Liver - preparation for quantitative culturing and microscopic examination

The livers received a special treatment in that they were homogenized in a Ten Broeck tissue grinder, cultured quantitatively or digested and examined microscopically. It was established in a preliminary test that grinding in the Ten Broeck tissue grinder did not damage M. alternans cells, single or budding. Strands of hyphae could not be expected, however, to go through the grinder undamaged.

For quantitative culturing, the homogenized livers of every third lot of mice were mixed with sterile melted 2% agar at 45° in such proportions as to obtain a final concentration of .5% agar, spread evenly over 4 plates of meat extract-cerelose agar, and incubated at 25° for 3 weeks. In the early part of experimentation, one portion of the liver homogenate was plated out and incubated as above, while the remaining portion was spread over the same medium in 250 ml Erlenmeyer flasks. The flasks were stoppered with rubber stoppers (same apparatus as was used in the in-vitro experiments), and connected to a cylinder of carbon dioxide-nitrogen (5% and 95% respectively), at 37°. The gas was flushed through the system at the rate of 120 ml/min for 60 min. The gas supply was then shut off,

the rubber tubings pinched off, the flasks disconnected and incubated on the shelf for 3 weeks at 37°.

Microscopic examination of livers, of the remaining 2/3 of the mice, was carried out in a dual fashion: the homogenization was carried out either at once after autopsy, or was delayed by about 48 hours. In either case the homogenate was digested with pepsin, 100 ml of 1% pepsin (Difco 1.10000) in 0.7% HCl-saline were used to digest one liver homogenate. As an extra precaution, 5 ml of 1% actidione were added to the digesting suspension to control fungal contaminants. The digestion was carried out at 37° for 90 min. with constant stirring with a magnetic stirrer. The suspension was then centrifuged at 350 x G for 30 min., the pellet resuspended in lactophenol cotton blue, and centrifuged in an International Clinical centrifuge for 1 hour. The sediment was washed twice in chloro-lactophenol and after the second centrifuging most of the supernatant was carefully pipetted off and the bottom 0.2 ml examined under the microscope. While the first 2 weeks' examinations were carried out on standard microscopic slides under a coverslip, all the remaining readings were taken with hemacytometers. When the homogenization was to be delayed, the excised whole livers were placed in sterile petri dishes and incubated for 1 hour at 37°. 2 hours at 25°, 3 hours at 15° and 15 - 18 hours at 4°, in that order.

The peritoneal cavity and the viscera were closely observed for any obvious granulomas or nodules. Whenever such structures or masses were observed, their incidence and location were noted, and the nodules excised for culturing and histopathological examination. In the latter case, the nodules were dropped into 10% buffered formalin. Sections of 3 nodules, weeks apart in their occurrence, were prepared by the kind co-operation of the Department of Pathology.

f) Blood glucose determinations and detection of urinary ketone bodies.

Glucose was determined using heart blood, by the Glucostat semi-micro method, the same one as used in the in-vitro experiments. Urines were tested for their ketone content by the Ketostix method (Ames).

The 5 pairs of mice injected with saline were sacrificed 2 pairs about half way through the experiment, and 3 at the end of the experiment and were processed in the same manner as those inoculated with the spores.

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IV - RESULTS

1.- In-vitro growth of Mucor alternans and M. subtilissimus under controlled gaseous conditions at 25° and 37°.

a) Growth of M. alternans and M. subtilissimus in YPG Broth under air and 100% nitrogen and carbon dioxide.

Gross morphology and microscopic features.

The results are presented in Table 1A. Under air, M. alternans grew predominantly in the filamentous form as a delicate light fluff under all conditions of incubation, i.e., in stationary and shaking cultures at 25° and 37°, and at the initial pH 4.5 and 6.8. Microscopic examination revealed the characteristic non-septate mycelium and arthrospore forming hyphae in all cultures. Large spherical single cells, about 10 to 25 microns in diameter, were also observed in most preparations. These cells could have arisen by step-wise fragmentation of the arthrospore chains (Fig. 12). The single cells were budding in cultures at 25°, more vigorously at the lower pH than at pH 6.8.

In shaking cultures under nitrogen, predominantly yeast-like growth was obtained at both pH levels at 25°, and at pH 4.5 at 37°. Growth at pH 6.8 at 37° appeared entirely filamentous. Microscopic examination of the predominantly yeast-like cultures showed vigorously budding cells and occasional hyphae. The single cells were typically spherical, or nearly so, about 10 - 20 microns in diameter, rather thick walled, with spherical buds. While

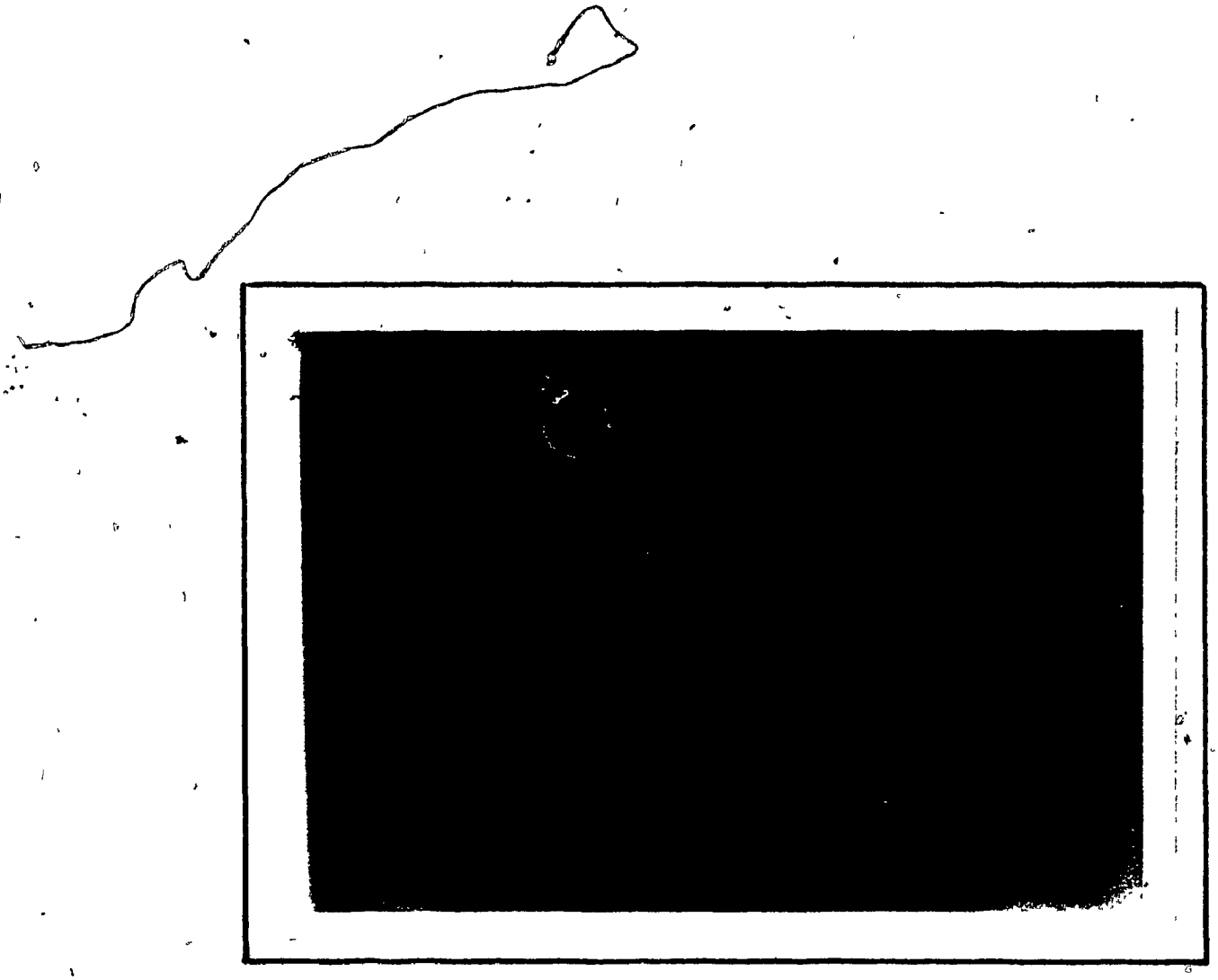


Fig. 12 - Arthrospore forming hypha from YPGb culture under air at pH 4.5 and 25° C.

multiple budding occurred most often, single budding cells were also observed. The buds were sessile on the mother cell and appeared firmly attached to it, since pressure applied to the coverslip failed to separate them. Similar spherical buds occurred on hyphae, particularly on the very broad and rather short ones. The filamentous growth at 37° at pH 6.8, revealed chains of arthrospores, indicating that given more time single and budding cells could appear in that culture also. The hyphae constituting the bulk of the mount were typically long and thin.

In shaking cultures under carbon dioxide, a purely yeast-like growth was obtained in all flasks. The microscopic features of the budding cells were similar in all respects to those from under nitrogen, and they are presented in Fig. 13.

M. subtilissimus grew only at 25°. Under air all cultures were typically filamentous and microscopic examination revealed, besides the characteristic non-septate mycelium, also arthrospore-forming hyphae. In shaking cultures at pH 4.5, occasional budding cells similar to those of M. alternans were also found.

In shaking cultures under nitrogen, and carbon dioxide, the growth of M. subtilissimus was almost entirely yeast-like resembling in every aspect of their morphology those previously described. A few hyphae were found under nitrogen at pH 4.5.

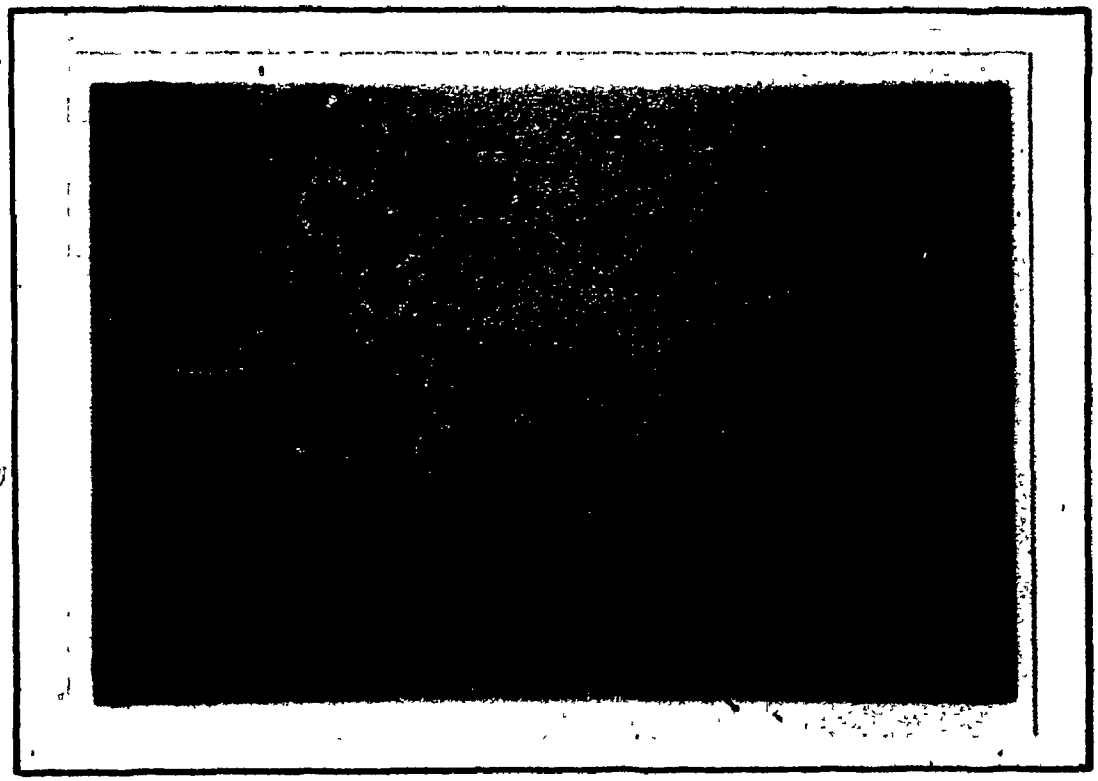


Fig. 13 - Yeastlike cells from YPGb, under 100% CO_2

Dry-weight determinations

The results are presented in Table 1B M. alternans grew well at both temperatures, but produced consistently more growth at 25 than at 37°C.

Incubation under air at 25° on the shaker, at pH 4.5, produced 224.0 mg D.W., or the greatest mass of growth in that series, while the parallel cultures incubated under stationary conditions produced 114.7 mg D.W., or only half that amount. This difference was more pronounced in cultures incubated at 37° where the amount of growth in the stationary cultures was only 1/3 of that obtained on the shaker.

At 25°, under nitrogen, M. alternans produced practically the same amount of growth (mean 91.5 mg D.W.), at both pH levels. Under carbon dioxide, an about equal yield (70.96 mg D.W.) was also obtained at both initial pH levels.

M. subtilissimus, as pointed out before, failed to grow perceptibly at 37°, the dry weight values varying from 0.1 to 0.6 mg.

Incubation under air on the shaker, at initial pH 4.5, produced 240.0 mg D.W., or the greatest amount of growth in that series, whilst its parallel cultures on the shelf produced 113.9 mg D.W., or less than half of that amount.

TABLE I

Growth of M. alternans and M. subtilissimus under Air, 100% Nitrogen and a 100% Carbon Dioxide.*

A. MORPHOLOGY OF GROWTH

Agitation and gas	T°	M. alternans		M. subtilissimus	
		pH 4.5	pH 6.8	pH 4.5	pH 6.8
Stationary Air	25	Y F A	Y F A	F A	F A
	37	F A	F A	-	-
Shaking Air	25	Y F A	F A	Y F A	F A
	37	F A	F A	-	-
Shaking Nitrogen	25	Y (F)	Y F	Y (F)	Y
	37	Y (F)	F A	-	-
Shaking Carbon Dioxide	25	Y	Y	Y	Y
	37	Y	Y	-	-

Y- yeast-like growth. F. filamentous growth. A: Arthrospore chains (an underlined letter indicates the predominance of that form of growth in the culture).
() very small quantity.

B. DRY WEIGHT DETERMINATION
(mg/50ml) total growth

Agitation and gas	T°	M. alternans		M. subtilissimus	
		pH 4.5	pH 6.8	pH 4.5	pH 6.8
Stationary Air	25	114.7	176.0	113.8	104.0
	37	53.4	56.0	0.3	0.2
Shaking Air	25	224.0	199.4	240.0	215.6
	37	173.0	157.4	0.2	0.1
Shaking Nitrogen	25	90.5	92.5	116.3	88.0
	37	60.5	42.2	0.3	0.6
Shaking Carbon Dioxide	25	71.4	70.6	64.0	36.5
	37	56.0	55.0	0.5	0.5

* Volume 50 ml. Time of incubation 67 hours

Incubation under all three gases showed a consistently greater yield at the initial pH 4.5, as compared to that at pH 6.8, the difference being particularly striking under carbon dioxide.

b) Growth of M. alternans in the TC Medium 199, with and without additives, under 5% carbon dioxide in aerobic and anaerobic gas mixtures.

Gross morphology and microscopic studies.

The results are presented in Table 2. Under aerobic conditions (carbon dioxide 5%, oxygen 13% and nitrogen 82%), either an entirely filamentous or a mixed yeast-like and filamentous growth was obtained. The filamentous growth was obtained in the basic medium (i.e., glucose 0.1%), and when urea, B-hydroxybutyric acid and acetoacetic acid, each at 0.1%, were added to the medium. The addition of glucose alone, to 1 and 2% to the basic medium, resulted in the appearance of single and budding cells. Addition of 0.1% each of B-hydroxybutyric acid and urea to the basic medium with glucose concentration, raised to 1%, seemed to have reduced the amount of the filamentous growth, but this was difficult to assess. Addition of the two acids and urea to the medium which had its glucose concentration raised to 2%, produced no apparent change in the quality of growth compared to that in the basic medium with glucose raised to 2%.

Under anaerobic conditions (carbon dioxide 5% and nitrogen 95%) pure yeast-like growth was obtained in the five tests performed.

Raising the glucose to 1 and 2% increased the amount of the yeast-like growth. Addition of urea and B-hydroxybutyric acid, both at 0.05 and 0.1%, reflected only the effect of the raised glucose concentration.

Microscopic examination of growth from the above cultures showed the characteristic spherical, multiple budding cells and hyphae with arthrospore chains indistinguishable from those observed earlier in the YPG broth experiments.

Dry-weight determinations

The quantitative evaluation of growth is presented in Table 2. Under aerobic conditions, the basic medium produced 16.8 mg D.W., while 15.1 mg D.W. were obtained, when the three non-glucose additives, each at 0.1%, were added to the basic medium. The raising of glucose concentration to 1% produced 98.5 mg D.W. but a further increase to 2% produced a surprising lower yield of 6.14 mg D.W. As observed before, the raising of glucose concentration caused the appearance of the yeast-like phase in the otherwise filamentous growth. Since each phase may have contributed a different weight load under different nutritional conditions, the over-all weight had to be affected correspondingly. (The two phases of growth were typically entangled and could not be reliably separated, the more so that buds often appeared along the hyphae, sometimes in considerable numbers).

TABLE 2

Growth of M. alternans in TC Medium 199 With and Without Additives, Under 5% Carbon Dioxide in Aerobic and Anaerobic Gas Mixtures *

Additives to TC Medium 199	Aerobiosis CO_2 5% O_2 13% N_2 82%		Anaerobiosis CO_2 5% N_2 95%	
	D.W. mg	Morphology of growth	D.W. mg	Morphology of growth
None	16.8	F	4.3	Y
Glucose to 1%	98.5	Y F (A)	25.4	Y
Glucose to 2%	61.4	Y F	36.4	Y
Glucose to 0.5% B-OH-but. a 0.05% Urea 0.05%	55.5	Y F	13.7	Y
Glucose to 1.0% B-OH-but. a 0.1% Urea 0.1%	82.5	Y F	25.2	Y
B-OH-but. a 0.1% Urea 0.1% Ac.-acetic a 0.1%	15.1	F		
Glucose to 2.0% B-OH-but.a 0.1% Urea 0.1% Ac.acetic a. 0.1%	57.1	Y F		

F: Filamentous growth Y: yeast-like growth A: arthrospores chains
() very small quantity. Underlined letter indicates predominance of growth

* Vol. 50 ml. T^{o} of incubation 37^{o} Time of incubation 48 hours Gyrotory shaker.

Under anaerobic conditions, the amount of growth was much smaller than in aerobic cultures. The growth in these cultures being uniformly yeast-like (Table 2), the determinations of dry weight paralleled clearly the increase in glucose concentration. Thus, while the basic medium (glucose 0.1%) produced 4.3 mg D.W., the raising of glucose to 1% yielded 25.4 mg D.W., and 36.4 mg when glucose was raised to 2%. The addition of B-hydroxybutyric acid and urea at 0.1%, to the basic medium with glucose at 1% yielded 25.2 mg D.W., showing that these two compounds did not affect the amount of growth produced.

C) Growth curve of M. alternans in TC Medium 199 with 1% glucose under 5% carbon dioxide in aerobic and anaerobic gas mixtures

On the basis of the results obtained in the preceding experiment, a growth curve of M. alternans was prepared in TC Medium 199, with glucose raised to 1% as the sole additive. The results are presented in Table 3 and Fig. 14.

After an initial lag phase of about 8 hours, the dry weight recorded in aerobic cultures at 24 hours showed a steeply rising curve which then continued to rise, but at a slower rate, until 123.4 mg D.W. were obtained at 67 hours. In these cultures, the pH fell slightly during the lag phase, from the initial level of 7.2 to pH 6.7 but fell sharply to pH 4.0 at 24 hr. The pH then started to rise slowly until it reached a value of 5.0 at 67 hr. These changes were accompanied by a drop in glucose concentration

to 0.46% at 24 hr. and 0.19% at 48 hr. At 67 hr no reliable reading could be obtained anymore, indicating that sometime between 48 and 67 hr. glucose was exhausted.

Microscopic examination of cultures at 8 hr revealed enlarged cells, some starting to bud, others to germinate. At 24 hr large spherical cells, about 10-20 microns in diameter, were observed, with several buds on each mother cell. Hyphae had elongated extensively and many had developed chains of arthrospores, the terminal spores showing a clear tendency for rounding off and separating from the rest of the chain. Similar growth was observed at 67 hr.

The anaerobic cultures behaved differently. The dry weight increased less than in the aerobic series, having yielded 20.3 mg D.W., at 24 hr, 24.9 mg at 48 hr, and 22.6 mg at 67 hr. The pH dropped gradually to 3.9 at 48 hr and remained there for the remaining 19 hr. Glucose decreased to 0,52% at 24 hr and already at 48 hr no further glucose readings could be taken. Microscopic examination revealed spherical enlarged cells at 8 hr, some of which had started to bud, and occasional cells with elongated buds. At 24 hr, 48 and 67 hr, all cells exhibited multiple budding, no hyphal growth at all being observed. The budding cells resembled those in the aerobic cultures.

On the basis of the above results, it can be concluded that the aerobic cultures showed a two-stage growth, a fast one

TABLE 3

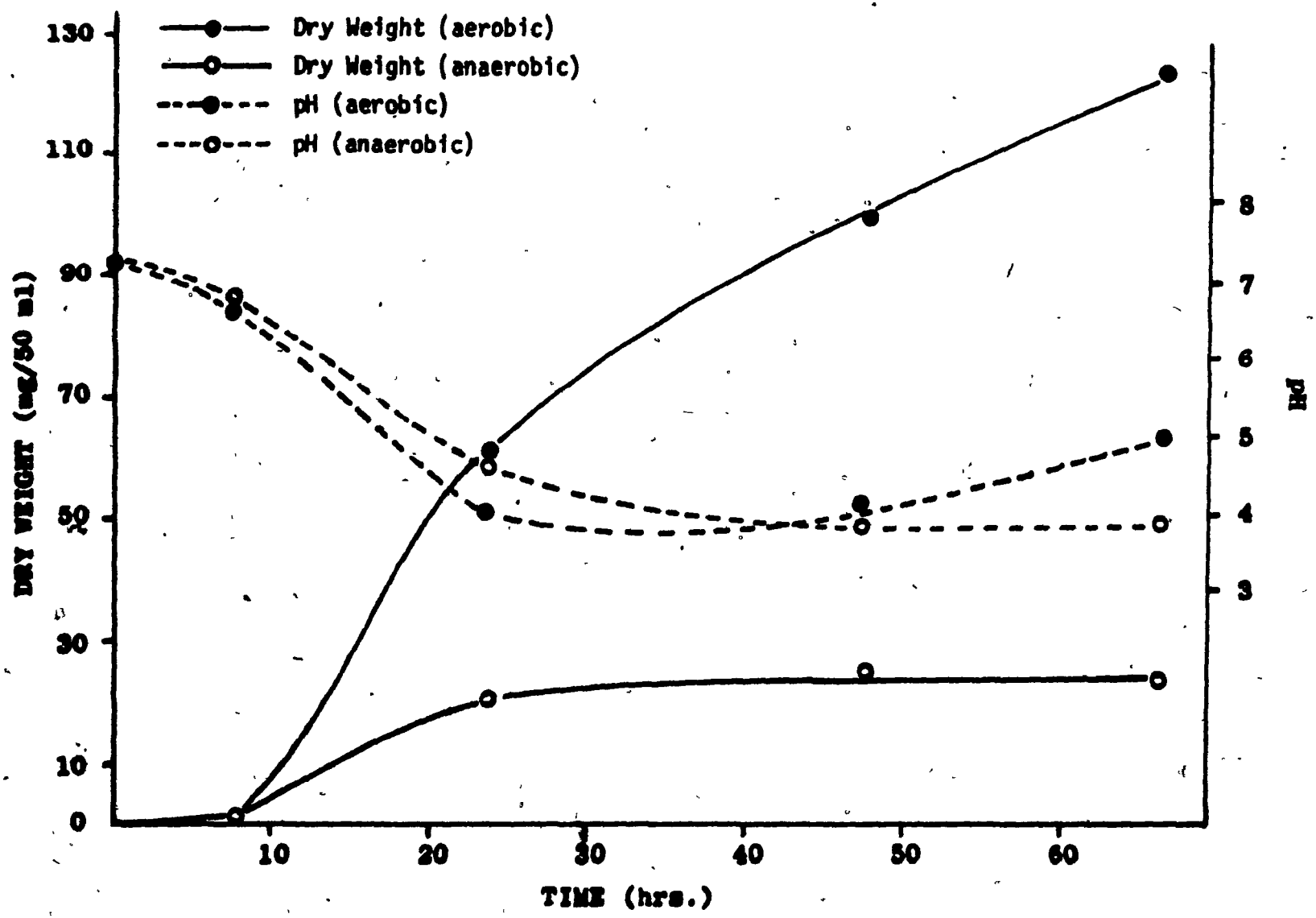
Growth of M. alternans in TC Medium 199 With 1% Glucose Under 5% Carbon Dioxide In Aerobic and Anaerobic Gas Mixtures **

Time Hours	AEROBIOSIS CO_2 5%, O_2 13%, N_2 82%				ANAEROBIOSIS CO_2 5%, N_2 95%			
	D.W. mg	Resid. Glucose %	pH	Morphology of growth	D.W. mg	Resid. Glucose %	pH	Morphology of growth
0	0.3	0.94	7.2	spores of inoculum	0.3	0.94	7.2	spores of inoculum
8	1.1	0.95	6.7	germinating and budding cells	0.5	0.85	6.8	enlarged cells some budding
24	61.1	0.46	4.0	mycellium and multiple budding cells	20.3	0.52	4.7	multiple budding cells
48	98.5	0.19	4.2	hyphae with arthrospores and budding cells	24.9	*	3.9	ditto
67	123.4	*	5.0	ditto	22.6	*	3.9	ditto

* reading beyond reliable range

** vol. 50 ml . . T° of incubation 37° on gyrotory shaker

FIG. 14 - Growth of M. alternans in TC Medium 199 with 1% Glucose



during the first 24 hours and a slow one during the following 43 hours, while the anaerobic cultures indicated a one-stage growth, which occurred between 8 and 24 hours of initial incubation.

2.- In-vitro growth of M. alternans from yeast phase inoculum on Sabouraud's agar under uncontrolled aerobiosis.

Since the proposed in-vivo experiments included incubation of some livers of the injected mice at gradually dropping temperatures, and a final exposure to 4^0, it was necessary to investigate the effect of the lower temperatures on the growth of this fungus in-vitro. Because the in-vivo experiments were to be carried out in imitation of diagnostic isolations, the media and methods of incubation in this and subsequent experiments were such as generally employed in mycological laboratories.

Yeast-phase inoculum was harvested from 50 ml YPG broth cultures (inoculated with 2×10^4 spores/ml and incubated for 67 hours at 37^0 under carbon dioxide), washed and suspended in 50 ml of sterile saline. Serial dilutions were prepared in saline and 0.5 ml aliquots used to inoculate plates of Sabouraud agar. Only the 10^{-3}, 10^{-4} and 10^{-5} dilutions were plated out. 12 plates were inoculated with each dilution and each lot divided into 4 groups which were incubated at different temperatures for 3 weeks, a) 4^0, b) gradually dropping temperatures, 37^0 for 1 hour, 25^0 for 2 hours,

15° for 3 hours, followed by 4° for the remainder of the incubation period, c) 37° and d) 25°.

The results of growth in the 10^{-4} dilution are presented in Table 4.

TABLE 4

Growth of M. alternans from yeast phase inoculum on Sabouraud agar under uncontrolled aerobiosis

T°	No. of fil.* colonies/plate (average of 3 plates)					
	24 hrs.	48 hrs.	5 days	8 days	14 days	21 days
4°	-	-	-	3.3	14	22
37 - 4°	-	-	12	23.3	27	overg.**
37°	22.3	25	25	overg.	overg.	overg.
25°	22	overg.	overg.	overg.	overg.	overg.

* fil. filamentous
** overg. overgrown

At 4° the first colonies appeared(3.3 /plate) at 8 days. The number of colonies increased gradually to 22 at 21 days, indicating that the cells inoculated were not all of the same germinating potential. The initial exposure of plates to gradually dropping temperatures during the first 7 days, hastened the appearance of growth by 3 days; at 14 days, 27 colonies were counted before they coalesced into one mass of light fluffy growth. Incubation at 37° produced a vigorous filamentous growth at 24 hr. At this temperature

the colonies remained clearly delienated from one another for several days and at 5 days, 25 colonies were still distinctly visible. Incubation at 25° produced the best aerial growth of the whole lot, which allowed a plate count only at 24 hr (22 colonies), thereafter the growth becoming completely coalescing and entangled.

Sporulation occurred abundantly at 25 and 37° in 24-48 hr. In the second group (dropping temperatures), growth in the 10^{-4} dilution sporulated profusely toward the end of the third week.

The above experiment showed that under ordinary aerobiosis M. alternans budding cells revert readily to the filamentous mode of growth, not only at room temperature (25°), but also at body temperature (37°), and even in cold storage (4°). The viable count varied little in the 4 series (22, 27, 25 and 22 col/plate), showing that the germination of the yeast-like cells is unaffected by temperature, although slowed down and delayed considerably by incubation in the cold.

3.- In-vivo experiments with mice inoculated with M. alternans

a) Physiological data on age, body weight, blood glucose and urinary ketones of the mice.

The mutated gene db was discovered in 1966 at Jackson's Laboratory in mice of the strain C57 BL/Ks. This gene determines

a metabolic disturbance resembling diabetes mellitus in man. The physiology of the diabetic strain C57 BL/KsJ-db/db has been investigated by Jackson's Laboratory. Homozygous diabetic mice, db/db, may be recognized at three or four weeks of age, due to their tendency to become obese. They increase in weight rapidly during the 2nd month, reaching a maximum weight of about 45 grams. Obesity, associated with overeating, is accompanied by hyperglycemia. Blood sugar concentrations of 300 mg per 100 ml at 4 weeks of age are not uncommon and by the time these mice are 12 weeks old, their blood sugar level is usually greater than 500 mg%. When the blood sugar concentration exceeds 250 mg per 100 ml, other symptoms, glycosuria, polyuria and polydipsia become evident. Normal mice have blood sugar levels ranging from 140 to 180 mg%.

The body weight of the mice recorded one day before inoculation, was as follows:

	Mice	age *	average body weight, g.
diabetic	(20	9 weeks	34.5
	(21	10 weeks	38.8
	(10	11 weeks	40.6
normal	(39	10 weeks	22.9
	(12	11 weeks	24.1

* Dates of birth varied from 1 - 3 days summarily within each group, according to information received from Jackson Laboratory.

During the 3 weeks after the first inoculation, as well as the two months following it, the mice appeared, apart from diabetes, normal in every respect, and none died, unless sacrificed.

Mouse No. 51, was more sluggish than others and was sacrificed ahead of schedule, but no evidence of infection or abnormality, except for a small skin lesion on the neck, was detected upon autopsy or tests. The age of the mice, their body weight, blood glucose and urinary ketone bodies at time of death, are presented in Tables 5A and 5B, for the diabetic and normal mice respectively The data have been arranged in groups of 5 for easier examination of the Tables.

In the 45 diabetic mice (Nos. 1 - 89), blood glucose was markedly elevated in nearly all the animals, although some had an unusually low level, e.g., mice No. 63 and 87 had 141 and 123 mg% sugar only. On the whole, the figures varied from mouse to mouse, without showing any increase upon aging. Data averaged for the seven age groups of 13 to 19 weeks, reflect similar variation in values, 461, 399, 437, 276, 394, 356 and 330 mg% respectively.

In the 45 normal mice (Nos. 2 - 90), blood glucose was much lower, as would be expected, although some readings were quite elevated, e.g., 391 and 209 mg% in No. 8 and 22 respectively. Values averaged for the seven age groups of 13 to 20 weeks, showed glucose content of 117,

TABLE 5A

Body Weight, Blood Glucose and Urinary Ketones of Sacrificed Diabetic Mice.

(The data are arranged in groups of 5 for easier examination of the Table)

Mouse No.	Age (approx. weeks)	Days after 1st inoculation	Body weight (g)	Blood glucose (mg %)	Ketone bodies
1	13	21	51	572	-
3	13	21	57	354	-
5	13	21	54	458	-
7	14	27	50	337	-
9	14	27	46	397	-
11	14	28	52	220	-
13	14	28	47	247	-
15	14	29	52	651	-
17	14	29	47	718	-
19	14	34	57	832	-
21	14	34	45	336	-
23	14	35	59	314	-
25	14	35	60	366	-
27	14	36	57	357	-
29	14	36	66	406	-
31	14	41	58	397	-
33	14	41	63	375	-
35	14	42	55	180	-
37	14	42	60	301	-
39	14	43	60	301	-
41	14	43	65	396	-
43	15	48	60	588	-
45	15	48	67	303	-
47	15	49	58	273	-
49	15	49	55	435	-
51	16	50	54	276	-
53	15	50	65	588	-
55	17	69	61	281	-
57	17	69	58	218	-
59	17	70	62	458	w.p.
61	17	70	61	521	-
63	18	71	60	141	-
65	18	71	58	300	-
67	18	76	62	694	-
69	18	76	61	694	-
71	18	77	56	304	-
73	18	77	62	245	-
75	18	78	62	187	w.p.
77	18	78	63	283	-
79	19	83	64	686	-
81	19	83	63	292	-
83	19	84	60	368	-
85	19	84	56	338	-
87	19	85	44	123	-
89	19	85	49	172	-
Saline inoculated controls					
C-1	16	52	57	391	-
C-3	16	52	56	234	-
C-5	18	59	62	334	-
C-7	18	66	53	157	-
C-9	18	66	35	482	w.p.

(-) negative

(w.p.) weakly positive, the lowest positive reaction on Ames chart

TABLE 5B

Body Weight, Blood Glucose and Urinary Ketones of Sacrificed Normal Mice

Mouse No.	Age (app. weeks)	Days after 1st inoculation	Body weight (g)	Blood glucose (mg %)	Ketone bodies
2	13	21	24	115	
4	13	21	27	87	-
6	13	21	30	149	-
8	14	27	23	391	
10	14	27	24	105	-
12	14	28	25	139	-
14	14	28	28	97	w.p.
16	14	29	27	347	
18	14	29	27	131	-
20	15	34	27	125	
22	14	34	26	209	-
24	15	35	27	140	
26	14	35	29	118	-
28	14	36	24	171	-
30	14	36	26	324	-
32	14	41	28	107	-
34	14	41	27	191	
36	14	42	28	87	-
38	14	42	24	126	-
40	14	43	25	102	-
42	14	43	26	102	
44	15	48	29	127	
46	15	48	27	114	
48	15	49	28	94	
50	15	49	23	118	

Mouse No.	Age (app. weeks)	Days after 1st inoculation	Body weight (g)	Blood glucose (mg %)	Ketone bodies
52	16	50	27	136	
54	16	50	27	131	-
56	18	69	32	143	
58	18	69	49	109	w.p.
60	18	70	28	152	
62	18	70	28	172	
64	19	71	29	83	-
66	19	71	24	196	-
68	19	76	27	115	-
70	19	76	28	112	-
72	19	77	27	244	
74	19	77	28	96	-
76	20	78	27	80	-
78	20	78	26	102	
80	20	83	30	64	
82	20	83	31	82	w.p.
84	20	84	29	66	w.p.
86	20	84	26	79	-
88	20	85	28	78	
90	20	85	28	108	w.p.
Saline Inoculated Controls					
C-2	16	52	25	139	
C-4	16	52	26	167	-
C-6	18	59	24	250	
C-8	18	66	25	99	-
C-10	18	66	28	136	-

(-) negative
(w.p.) weakly positive, the lowest positive reaction on Ames Chart.

172, 120, 133, 144, 141 and 70 mg% respectively.

Urines were collected from all but one diabetic mouse. 42 were found negative for ketone bodies and 2 were weakly positive. One of the two positive specimens was obtained from a highly diabetic mouse with 458 mg% of blood glucose, the others from a nearly normal mouse (187 mg%).

16 normal mice had emptied their bladders before or during the Nembutal injection, and no sample could be collected from them. 5 samples were found weakly positive and 24 negative. Since no ketone bodies were expected in the urines of normal mice, the batch of ketostix strips used was re-tested and revealed that the test strips were still good and had not deteriorated.

Saline inoculated controls: Between the 52nd and 66th day after inoculation, the 5 pairs of saline-inoculated controls were sacrificed. The average weight of the diabetic mice was 53 g and their blood glucose varied from 157 to 482 mg% (average value: 320 mg%). The average weight of the normal mice was 25 g and their blood glucose varied from 97 to 250 mg% (average value 158 mg%).

b) Qualitative cultures of organs and body fluids

The qualitative results of cultures are presented in Table 6. Only the diabetic mice produced positive cultures. Growth in the early

TABLE 6

Isolation of M. alternans from Cultured Organs, Blood, Urine and Nodules of Sacrificed Mice.

	Mouse No.	Liver	Heart	Lungs	Spleen	R. Kidney	L. Kidney	Brain	Blood (heart)	Urine	Peritoneal nodules positive only
DIABETIC	1	+	-	-	+	+	-	-	-	-	
	3										
	5										
	7	+	+		+						
	9	+		+							
	11	⊕			+						
	13										⊕
	15				+			+			
	17	⊕									⊕
	19										⊕
	21										⊕
	23										⊕
	25										⊕
	27	⊕			+						⊕
	[redacted]										⊕
	31	-									⊕
	33										
	35										
	37										
	39										
	41										⊕
	43										
	45	-									⊕
	47										
	49										
	51										
	53										⊕
	55-89	-	-	+	-	-	-	-	-	-	
Normal	2-90	-	-	-	-	-	-	-	-	-	

\+ or - positive or negative cultures of organs on slopes or liver on plates

⊕ positive cultures of excised nodules.

positive cultures of organs on slopes and of liver homogenates under uncontrolled aerobiosis at 25°, appeared as a vigorous mycelium in 24 - 48 hr. Later it took 3 - 4 days before the cultures became positive, denoting a gradually impaired viability of the fungus in the tissues. Once started, the growth soon became abundantly bulky, grey with the production of sporangia, filling eventually the entire tube or plate. Altogether 17 out of the 45 diabetic mice, or 37.7%, were positive in cultures, (this figure includes positive cultures from nodules). The last positive culture was obtained in mouse No. 53, which was sacrificed 7 weeks after inoculation. Liver was the organ most frequently positive (6 mice), spleen was positive in 5 mice, while lungs, heart, brain and right kidney were positive, each once in 4 mice. Except for mouse No. 15, from which the liver was not cultured, all positive organs occurred in mice that had a positive liver culture. Heart, blood, urine and left kidney were all negative in culture. Occasional cultures of peritoneal washings were negative.

c) Quantitative cultures of homogenized livers.

Quantitative cultures of livers (Table 7), yielded data from only 3 out of 15 mice so investigated, No. 1, 7 and 9 from which 56, 34 and 32 colonies respectively were obtained. The quantitative flasks incubated under anaerobiosis, produced pasty white, rather flat, yeast-like colonies with irregular margins,

consisting of large spherical multiple budding cells and chains of linked cells, presumably arthrospores (Fig. 15). These colonies resembled closely those of M. rouxii observed by Bartnicki-Garcia and Nickerson (1962 b), under anaerobiosis. Two of the three anaerobic flasks produced a few filamentous colonies of M. alternans on the glass of the flasks from inoculum smeared accidentally just above the agar line, indicating that should some vital nutrients fall below a certain minimum, M. alternans would revert to mycelial growth even under anaerobiosis. Although more colonies were obtained in the anaerobic flasks than on aerobic plates, the data are insufficient to comment upon, particularly that the thick inoculum did not allow a strictly quantitative distribution.

TABLE 7

Number of Colonies Obtained From Homogenized Livers of 3 Diabetic Mice.

Mouse No.	Aerobic plates (25°)					Anaerobic plates (37°)	Total Colonies
	1	2	3	4	5		
1	16	13	13			14	56
7	7	1	4	6	4	12 *	34
9	4	3	5	9		11 +	32

* Includes 5 filamentous colonies on glass

\+ Includes 2 filamentous colonies on glass

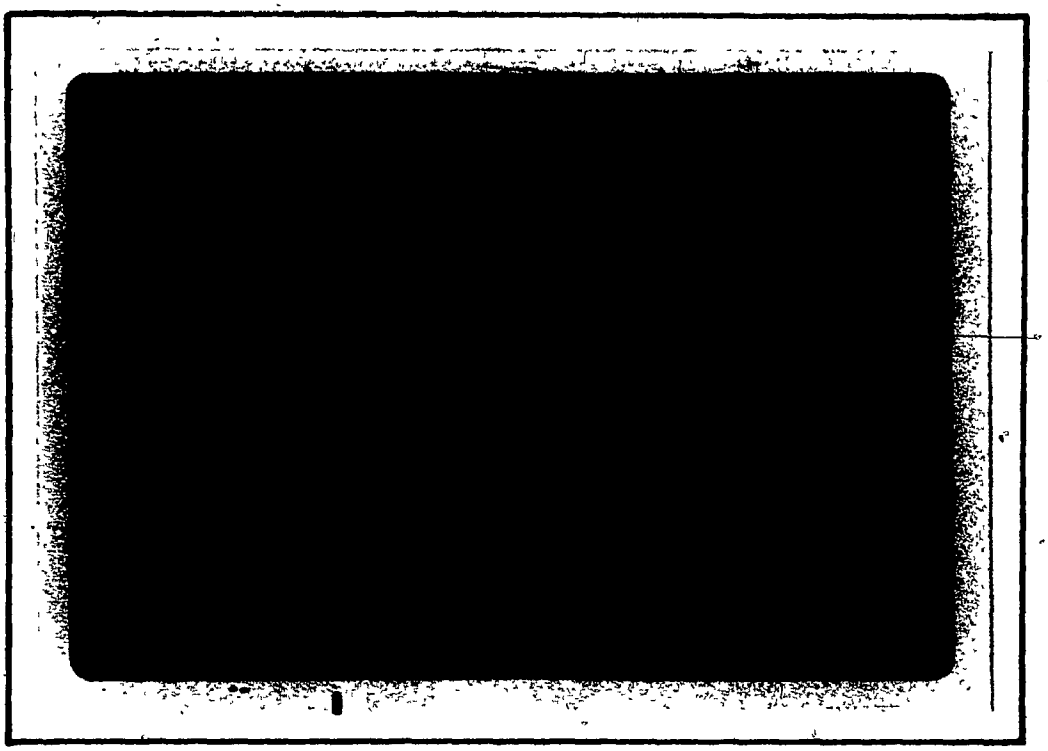


Fig. 15 - Lactophenol cotton blue stain of cells from yeastlike colonies obtained from incubation of liver homogenate under a mixture of 5% CO_2 and 95% N_2 at 37^{o} C.

Normal mice and saline-inoculated controls were all negative in culture. It should be borne in mind, however, that only 1/3 of the livers were cultured throughout this study, and it is quite possible that mouse No. 6, whose liver showed on microscopic examination (Figs. 16 and 17), numerous budding cells, and vigorous hyphae with buds, would have produced positive cultures if such had been prepared.

d) Nodules - their occurrence and culturing.

The first nodules were observed in mouse No. 11 which was sacrificed 28 days after inoculation. Thereafter, nodules became a common occurrence and thus their numbers and location were noted. 80 nodules were found in 36 diabetic mice, and 12 in 8 normal mice. No nodules were observed in the saline-inoculated controls. A specific occurrence of the nodules is presented in Table 8

TABLE 8

Location and incidence of nodules

Location	Incidence in Diabetic mice 36/45	Normal mice 8/45	Saline controls
in/on liver	16 *	6 **	0
in peritoneal fat	44	3	0
on omentum	9	1	0
at site of inoculation	4	1	0
near bladder	3	0	0
on peritoneal wall	2	1	0
on diaphragm	2	0	0

* in 11 mice
** in 5 mice

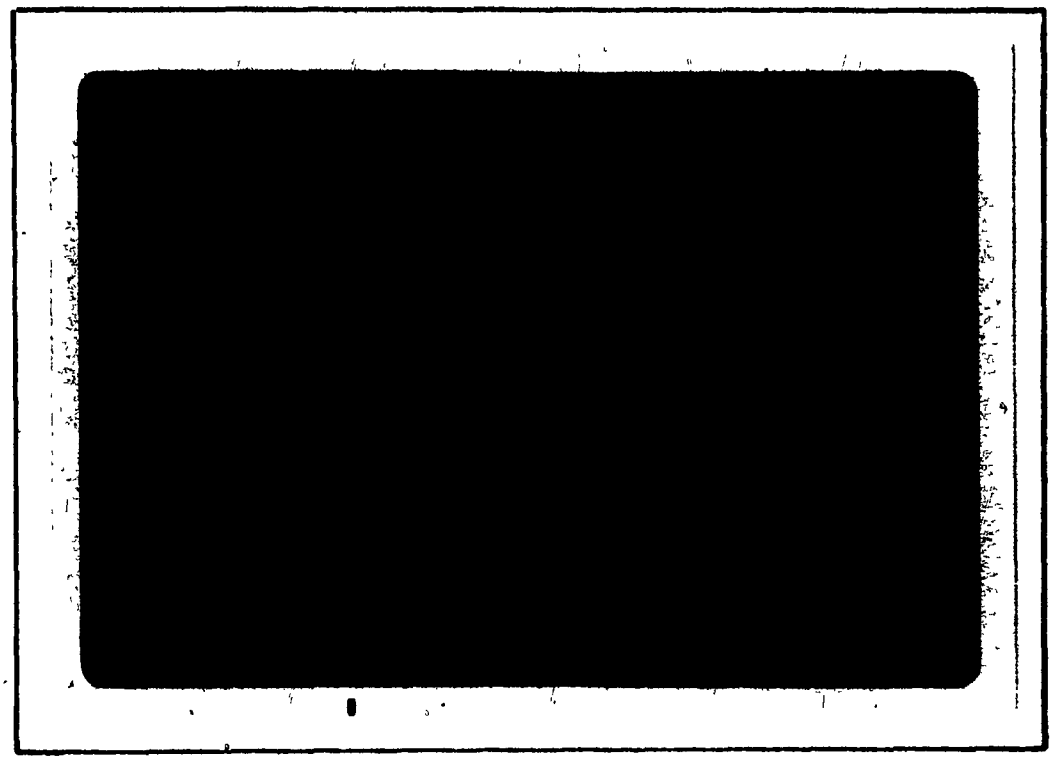


Fig. 16 - Budding cells from homogenized pepsin digested liver of mouse No. 6. ✱

Fig. 17 - Hyphae forming arthrospores: two chains of arthrospores obtained in liver homogenate of mouse No. 6.

The first nodules encountered in the diabetic mice were rather soft and measured about 2 mm. The older nodules became progressively smaller, and harder, as if shrinking, and looked like tiny grains of sand. The nodules in the normal mice were generally smaller and harder than those in the diabetic mice.

In terms of age, the greatest number of nodules was observed in younger mice sacrificed between the 35th and 50th day after inoculation.

Liver nodules received most of the attention. While some nodules were visible to the unaided eye during autopsy, others were discovered accidentally inside the liver when the subsurface nodules were being examined. Thus, the number of nodules recorded in livers may, in fact, represent only a fraction of the total number of nodules occurring in that organ.

Upon culturing, most of the nodules found in diabetic mice sacrificed between the 28th and 35th day after inoculation, produced typical cultures of M. alternans. Only occasional nodules excised from the older diabetic mice were positive in culture. The last positive culture altogether was obtained from a nodule in the peritoneal fat of mouse No. 53. Nodules from normal mice were all negative in cultures.

e) Microscopic examination of homogenized and pepsin-digested livers.

Microscopic examination of liver digests in wet smears, was at first a truly rewarding experience. Cotton-blue stained spherical budding cells and, on rare occasions, large, non-septate hyphae, conforming in every respect to those of M. alternans, were seen clearly in many preparations. Furthermore, the slides remained in good condition for several months upon storage in a tightly sealed glass dish before the first one could be photographed. Preparations made of livers of aging mice were less satisfactory; artifacts in form of spherical to oval membrane-bound structures, some 5 - 50 microns in size, started to occur in quantity obstructing the search for fungal elements, resulting in 5 pairs of preparations being discarded as unreliable.

The results of this rather fragmentary investigation allow only broad generalizations:

1) Both diabetic and normal mice were found microscopically positive, the diabetic mice producing more positive preparations than the normal ones.
2) Mostly non-budding cells were observed. Collapsed and broken-walled cells were observed in most preparations and their proportion seemed to have increased in older mice. Germinating cells and hyphae were found in only 7 diabetic and one normal mouse.

3) There was no obvious difference in morphology of the fungal elements found in livers pepsin-digested at once after autopsy, and livers incubated at dropping temperatures prior to homogenization and pepsin digestion.

Quantitative estimation of cells with hemacytometer was done in 23 diabetic and 24 normal mice. Because of the low incidence of the cells, not the R's but whole square millimeter grids were examined, the large size of the cells, about 5 - 25 microns, allowing a good count in most cases. The entire sedimented liver preparations were used up in these examinations, the 0.2 ml sufficing to cover on the average 10 coverslip areas. The results are presented in Table 9.

TABLE 9

The incidence of M. alternans cells in livers

Total cells per liver	Diabetic Mice (23)	Normal Mice (24)
0	3*	12
<2,000	12	9
2,000 - 10,000	4	3
10,000 - 40,000	3	
64,400	1	

* One of these 3 mice, No. 17, had a liver nodule which was excised and cultured (the culture was positive, see Table 6). The liver was then homogenized, digested and generally prepared for microscopic examination.

The incidence of fungal cells in diabetic mice was lower in older mice, including the two negative readings, in mice No. 51 and 89, i.e., the majority of the <2,000 cell group occurred in mice No. 53 to 87, although, contrary to that trend, the highest single reading recorded, of 64,400 cells, was obtained from an older mouse No. 71. In the normal mice too most of the low readings were obtained from the older mice.

Mouse No. 6 deserves a special comment. The preparation was examined qualitatively only on standard microscopic slides. Very numerous cells, spherical, single and surrounded by numerous buds, as well as hyphae with arthrospore chains, were found in many fields, as illustrated in Figs. 16 and 17. Because the budding cells often formed large clusters while hyphae occurred in bundles, no count would have been possible (this mouse, being investigated qualitatively only was not included in Table 9).

f) Histopathological study of nodules.

Histopathological sections of a nodule from a mouse sacrificed 29 days after inoculation (No. 17), revealed yeast-like cells, some so large as to simulate small blood vessels of a mouse in a hematoxylin eosin stained section. Many of these cells were damaged, i.e., their cells were split open and they appeared empty. Some smaller cells were in apparently good condition. Budding and

germinating cells were also found rather infrequently (Figs. 18, 19 and 20). A few hyphae were seen in some sections and could easily be identified in Grocott stained slides (Fig. 21). The PAS stained sections of this nodule showed granuloma formation with no giant cells but with lymphocytes and plasma cells on the periphery. The center of the nodule was composed of cells debris, necrotic tissue and a few blood vessels. This was surrounded by collagen tissue and histiocytes. Nodules from older mice (Nos. 23, 27, 39 and 83) showed a burnt-out condition without any fungal cells in evidence.

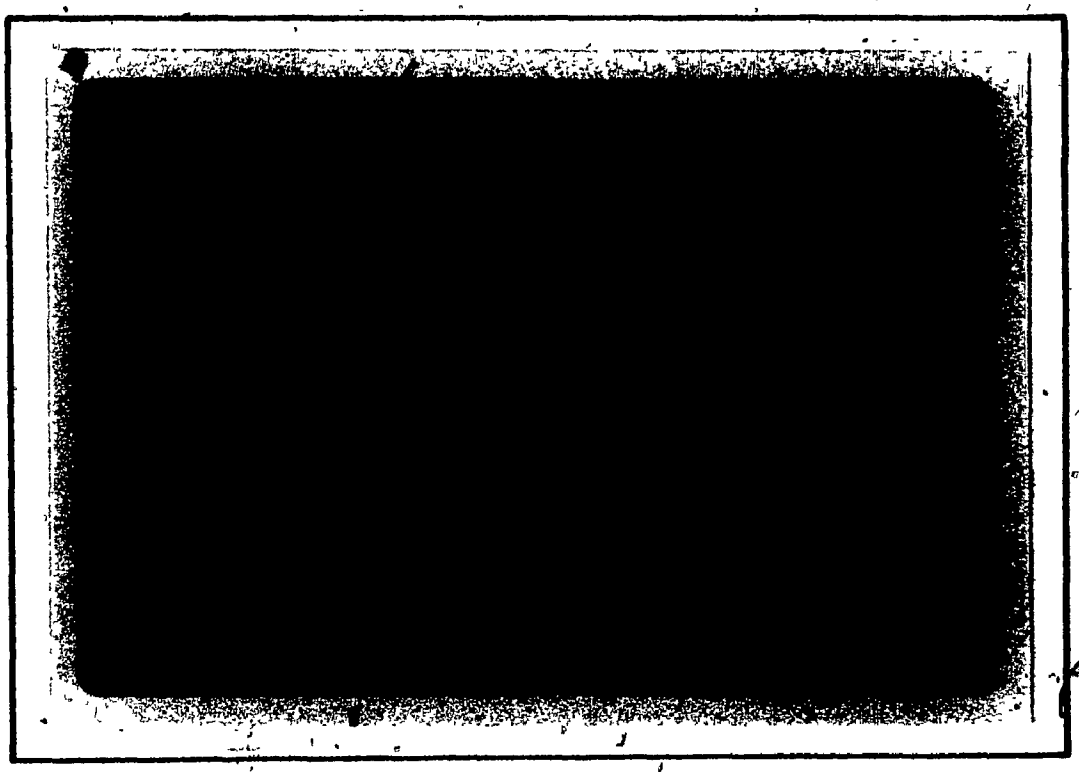


Fig. 18 - Single cells and budding cells in section of a nodule from mouse No. 17. Stained with PAS.

Fig. 19 - Budding cell in section of a nodule from mouse No. 17. Grocott staining.

Fig. 20 - Germinating cells in section of a nodule from mouse No. 17. Grocott staining.

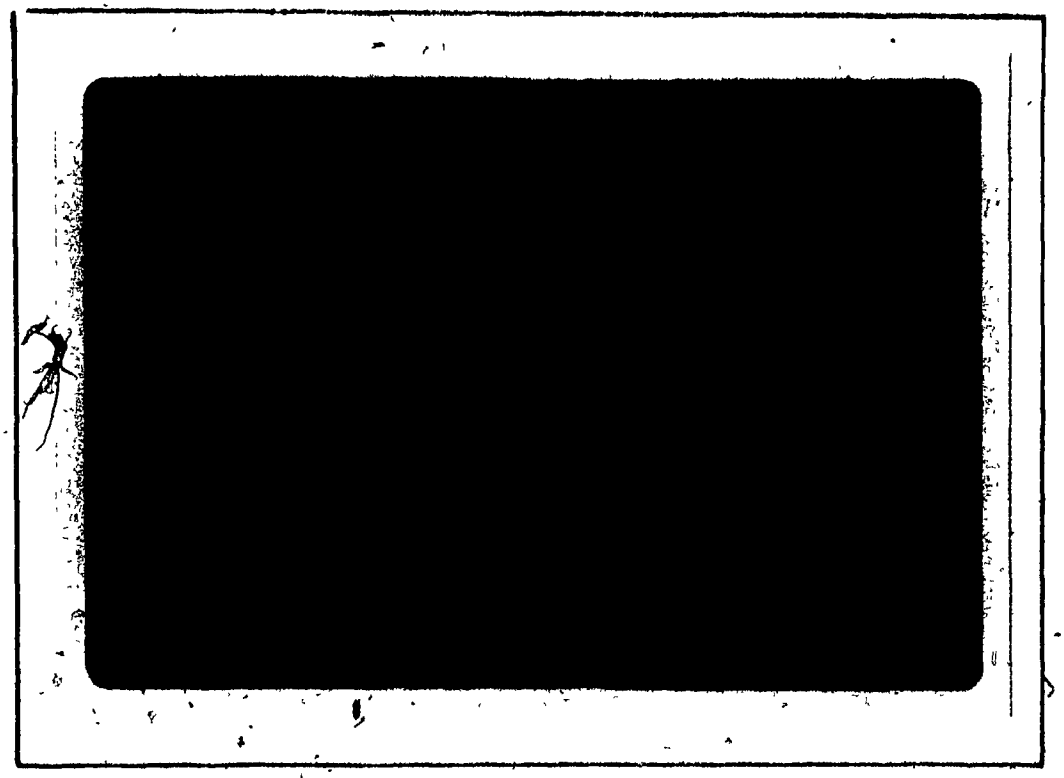


Fig. 21 - Piece of hyphae in section of a nodule from mouse No. 17. Grocott staining.

V - DISCUSSION

The ability of Mucor alternans to form unicellular budding cells was first observed in sugar-rich media by Gayon and Dubourg in 1887, and a description of this type of growth has been quoted at length in the Review of Literature. The remarkable fermentative powers of this species attracted the attention of Japanese researchers who deposited strain No. 20132 in the American Type Culture Collection (10th ed. catalogue), following an application for a patent by Kyowa Ferm. Ind. Co. Ltd. Otherwise, M. alternans is rarely being mentioned in the literature, and does not appear in textbooks of general or medical mycology. Neither did the Bartnicki-Garcia and Nickerson group include this species in their studies of dimorphism in Phycomycetes. Its occurrence under apparently pathological conditions at the Royal Victoria Hospital, Montreal, in 1962, has prompted an investigation of its dimorphism in-vitro and of the pathogenicity of its yeast-like phase for diabetic mice.

The in-vitro studies were carried out under a variety of conditions. Under aerobiosis in the yeast extract-peptone-glucose (YPG) broth, M. alternans grew in the filamentous form. The fact that more growth was obtained in the shaking cultures as compared to stationary ones is not surprising since fungi are basically aerobic organisms, they produce more growth with better aeration, while the

mechanical agitation per-se aids growth by a faster distribution of nutrients in the medium. Cells of the yeast-like type were seen in some of these cultures, but since arthrospore formation is characteristic of this species, it could not be ascertained if the observed budding cells arose directly from the sporangiospore-inoculum or from broken-off arthrospores formed on mycelium. Bartnicki-Garcia and Nickerson (1962 b), emphasized the fact that true yeast-like cells, microscopically, may be indistinguishable from spherical cells derived by arthrosporal morphogenesis.

Under anaerobiosis only the YPG cultures at 25^0 at pH 4.5, or conditions under which Bartnicki-Garcia and Nickerson (1962 b) found M. subtilissimus to grow in pure yeast-like form, can be considered for comparative studies of M. alternans.

Under nitrogen, M. alternans and M. subtilissimus produced almost identical appearing cultures, i.e., both were yeast-like, except for very small amount of filaments. Nutrition may have played a part in this experiment since in both species the filaments were formed primarily on the glass where some spores might have been splashed on during the experiment. Since growth on the glass was poorly supplied with the medium, some vital nutrients were probably lacking for initiation or maintenance of the yeast-like growth.

Under carbon dioxide a pure yeast-like growth was obtained in both species.

The above results thus showed that M. alternans is a diphasic species in that under air it grows in its typical filamentous form and under anaerobiosis it assumes a yeast-like form of growth.

Since the potential pathogenicity of M. alternans constituted the main object of this study, the in-vitro investigation in YPG broth was extended beyond Bartnicki-Garcia and Nickerson's scope to include incubation at 37^0, i.e. body temperature. The results showed that while M. subtilissimus grew only at 25^0, and not at 37^0, thus proving that this species could not be pathogenic, M. alternans generally grew well at 37^0, indicating that in terms of temperature this species could be pathogenic. However, in contrast to pathogenic diphasic fungi, such as Histoplasma capsulatum or Blastomyces dermatitidis temperature influenced rather the amount of growth, as shown by dry weight determinations, than the growth form. In all trials more growth of M. alternans was produced at 25^0 than at 37^0.

In the TC Medium 199, under a gas mixture imitating alveolar atmosphere, i.e., consisting of 5% CO_2, 13% O_2 and 82% N_2, growth was predominantly filamentous while increasing concentrations of glucose favoured the development of yeast-like growth. A critical inspection of Table 2, shows a rather odd set of data under dry weight, the value for growth in a 2% glucose-enriched medium being less than at 1%, 61.4

and 93.5 mg respectively. It is possible that the yeast-like cells contributed less weight because of their slower growth rate as compared to that of the filamentous form. The difference in the rate of growth of the two phases was reported by Bartnicki-Garcia and Nickerson (1962 b), for M. rouxii, and confirmed by my own observations for M. alternans in a preliminary experiment. When ketones and urea were added to glucose-enriched medium, the dry weight became a little smaller while morphologically one of these cultures changed from a predominantly filamentous to a predominantly yeast-like form. (It should be borne in mind that a qualitative evaluation of growth from microscopic slides is bound to be subject to considerable error).

Under anaerobic conditions of 5% CO_2 and 95% N_2, a pure yeast-like growth was obtained, increasing glucose concentrations producing increasing amounts of growth. It appears that addition of B-hydroxybutyric acid and urea had no effect upon growth under these conditions, the dry weight values reading 25.4 mg without additives, and 25.2 mg with additives.

The growth of M. alternans in 1% glucose-enriched TC Medium 199, produced under aerobic conditions a seemingly two-stage curve, the amount of the mostly filamentous growth increasing over the 67 hr of the incubation time. Under anaerobiosis, the exclusively yeast-like growth increased over the first 24 hours only, and then levelled off. The study of the pH in this experiment, indicated that the medium being

insufficiently buffered, allowed the pH to drop during growth by as much as about 3 units. Under aerobiosis, the pH eventually rose to 5 at 67 hr showing that the fungus started to utilize the products of glucose catabolism, which would constitute the second phase of that growth curve. Under anaerobiosis, however, the pH remained at its lowest level to the end of the incubation time, indicating that in the absence of oxygen, M. alternans cannot utilize the acidic products of glucose breakdown.

Yeast-like cells of M. alternans produced under CO_2, reverted, upon incubation in air, to the filamentous growth at all temperatures tried, although at different times. At 4^{o}, filamentous colonies were observed with unaided eye at 8 days. At 25 and 37^{o}, within 24 hours, the colonies were visible while on plates incubated at the dropping temperatures from 37 to 4^{o}, Mucor grew visibly at 5 days. (In terms of organs stored prior to fixation, the above experiment carried out, as it was, in air, could perhaps explain a possible post-mortem growth of fungal hyphae at cut surfaces. However, such saprophytic growth could easily be differentiated by the pathologist from one which occurred in-vivo).

The answer to Question 1 can thus be formulated as follows: M. alternans produced an exclusively yeast-like growth under carbon dioxide at 25 and 37^{o}, at an initial pH value of 4.5 and 6.8 in the non-defined medium of yeast-extract-peptone-glucose broth and under

a mixture of carbon dioxide and nitrogen (5 and 95% respectively) at 37°, and initial pH 7.2 in the defined tissue culture medium 199. The amount of growth in the TC Medium 199 depended on glucose concentration, the yeast-like growth having been obtained even at the lowest glucose concentration of 0.1%. The yeast-like cells revert to mycelial growth when exposed to air. (Growth in the YPG broth under nitrogen requires further experimentation).

In-vivo experiments with diabetic mice

In 1966, Jackson Laboratory (Hummel et al. 1966), reported that their strain of diabetic mice (C57 BL/KsJ-db/db) succombs to diabetes in 3 - 6 months, with accompanying loss of weight. The mice received from that Laboratory in December 1972, remained in remarkably good condition during the 5-month long experiment. None died (unless sacrificed), and no noticeable weight loss was observed. The average weight of the diabetic mice (13-19 weeks old) was 57 g. which was higher by 12 g than the 45 g reported by the Laboratory. Although blood sugar was as high as expected in this strain, it varied greatly from mouse to mouse throughout the lot, from about 123 (a normal value) to 832 mg%, without pattern or trend. No mice were truly ketotic, only two showing a weakly positive reaction, the lowest on Ames chart. The saline-inoculated controls showed a similar span of body weight, blood glucose and ketones. Normal mice on the whole conformed better to the norms described by the Laboratory.

except that 4 mice had an elevated blood glucose, i.e., about 200 mg% and 5 gave a weakly positive test for urinary ketones. The normal mice were identified by the Laboratory as -/? to denote uncertain information about their genetic character and this may account perhaps for the rather weak diabetic tendencies in this particular lot of animals.

The Mucor-inoculated mice, diabetic and normal, remained grossly asymptomatic throughout the experimental period. The examination of the peritoneal cavity and the organs, however, revealed that invasion of tissues did occur and the fungus was re-isolated from 37.7% of diabetic mice. Liver was the organ which was most often positive in culture, followed by spleen, while lungs, heart, kidney and brain each produced one positive culture. Urine, blood and an occasional peritoneal washing were all negative in cultures. The fact that peritoneal washings were negative proved that positive cultures of the organs were not due to contamination with spores surviving within the peritoneal cavity. The positive organs from the youngest mice, i.e., those killed 3 - 4 weeks after inoculation, appeared normal upon autopsy. Later, nodules were observed on the peritoneal wall, omentum, in the peritoneal fat and on the liver. Eventually, nodules were discovered deep in the liver tissue. Characteristically, such nodules carefully excised, produced positive cultures while the nodule-free livers were negative upon microscopic examination, indicating strict localization of infection.

Altogether 80% of diabetic mice exhibited nodule formation. At first, most of the nodules were positive in culture, but gradually the cultures became negative and, starting with the 50th day after inoculation, all attempts at culturing the fungus failed, showing that the mice succeeded completely in recovering from the infection.

All cultures prepared from normal mice were negative. 17.7% of normal mice exhibited nodule formation, but like the organs the nodules were negative upon culturing.

Cultures of the livers of the first three diabetic mice produced yeast-like colonies under anaerobiosis, indicating that this method of incubation could yield positive cultures for diagnostic purposes. Although incubation under anaerobiosis is hardly a method of preference in mycological laboratories, bacteriological technicians working with anaerobes should perhaps be alerted to such a possibility.

Thus Question 2, of this study, can be answered in the following way: M. alternans is pathogenic in that the disease was produced in diabetic mice, but the mice did not succomb to it. In agreement with Bauer (1956 b), hyperglycemia alone did not favour the development of a fulminating infection. Even if ketosis had developed in the 5th month, the fungal cells in the nodules being already dead, no further developments could be expected. To attain a fatal issue, a close coincidence of the presence of viable Mucor cells and opportunistic physiological changes, such as ketoacidosis (Bauer and Sheldon 1958), must occur. In diabetic mice such coincidence

should occur within 7 weeks after inoculation, but preferably earlier, i.e., within 4 weeks after inoculation, before the formation of nodules. The second part of Question 2, can be answered in affirmative - normal mice succeeded in eliminating the infection, in that none of the so-tested mice was positive in cultures, re-affirming the opportunistic nature of mucormycosis.

Microscopic examination of homogenized and pepsin-digested livers, revealed that both diabetic and normal mice were positive for fungal elements, the diabetic mice showing, except in one case, many more cells than the normal ones. Many spherical single cells were observed; budding cells were also encountered, while germinating cells and hyphae were observed very rarely. Collapsed and broken cells were also observed indicating destruction by the host.

The best results of direct microscopic examination were obtained, ironically, in a normal mouse (No. 6), whose liver revealed the greatest number of fungal elements recorded in this study, in any single test. Extensive hyphae with arthrospore chains, as well as multiple-budding cells were observed in clusters, all in perfect morphological condition. Obviously, an unusually large and apparently still active focus was encountered by chance, in this particular young mouse. Now that a fine technique has been worked out, future studies can concentrate on direct examination of livers prior to formation of nodules in young mice, as the most promising approach toward a more precise elucidation of the mode of dissemination in phycomycosis.

Upon microscopic examination of histopathological sections of a nodule from mouse No. 17, many damaged and a few undamaged cells were seen. Budding and germinating cells were also found, although rather infrequently, which confirmed the microscopic studies of digested livers. A closer inspection revealed a few hyphae in most sections, proving that conditions inside the nodules allowed mycelial growth rather than active budding. Nodules from older mice showed a burnt-out condition without any fungal cells in evidence, also confirming microscopic examinations which were mostly negative in older mice.

The answer to Question 3 has been carefully formulated as follows: due to a restricted number of mice used in this study, only a tentative conclusion can be drawn. M. alternans mycelium can produce arthrospores in-vivo as it did in mouse No. 6. After breaking off, the arthrospores can be visualized as single cells, disseminating the disease, further growth of the fungus occurring by germination rather than budding, as observed in the histopathological sections of the nodule of diabetic mouse No. 17.

Answer to Question No. 4 appears to be negative. No evidence has been found to substantiate the supposition of post-mortem filamentous growth. The [redacted] in mouse No. 6 had occurred in liver which was processed at once after autopsy. Microscopic examination of all other mice, failed to produce any signs of morphologically

different fungal elements, in the two methods employed in the handling of the livers.

Experimentation with immuno-suppressive drugs may bring about a fulminating course in mucormycosis, and provide chances of positive cultures from body fluids. Since mucormycosis is par excellence an opportunistic disease, future work should concentrate on immunological compromised hosts, whether by therapy as in transplantation patients or patient under treatment of cancer with cytotoxic drugs, or by lack of therapy, as in uncontrolled ketotic diabetes.

Too little information is available, unfortunately, about the identity of the specific causative agents of mucormycosis. In many instances, only a post-mortem diagnosis is made from histopathological sections, which does not allow more than a vague description of a phycomycetous fungus, while the occasional reports of isolations in mycology rarely go beyond identification of a genus. Thus it is not known whether M. alternans, isolated from the debris of torn tissues in the patient at Royal Victoria Hospital in 1962, is or is not a commonly occurring pathogenic species. Until greater care is taken in isolation and identification of phycomycetous fungi, from opportunistic infections, no answer to this question is possible. Identification of species within that group of fungi is no easy matter, and smaller laboratories ought to seek help from researchers experienced

in this domain of mycology. As acknowledged in Materials and Methods, M. alternans isolated at the McGill Mycological Laboratory was so identified by the kind and expert assistance of Dr. Hesseltine, Peoria, Ill.

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VI - SUMMARY

1) Filamentous growth of Mucor alternans was seen upon incubation under air. Exclusive yeast-like growth was obtained under carbon dioxide at the two temperatures investigated, i.e., 25 and 37°, and at an initial pH value of 4.5 and 6.8 in yeast-extract-peptone-glucose broth. This type of growth was also observed under a mixture of carbon dioxide and nitrogen (5 and 95% respectively), at 37°, and initial pH 7.2 in the defined tissue culture medium 199. It was also observed that the yeast-like cells reverted to mycelial form when exposed to air.

2) When diabetic mice were injected intraperitoneally with preincubated spores of M. alternans, there was dissemination of the fungus to internal organs, sometime during the first weeks after inoculation, which was rendered evident by positive cultures obtained from visceral organs, lungs, heart and brain. At 4 weeks, nodules started to appear in various parts of the peritoneum, mainly in the peritoneal fat and liver. Until 50 days after inoculation, the excised nodules gave positive cultures, indicating localization of the infection. From then on, organs and nodules remained negative in culture. The diabetic mice overcame the infection. Some nodules were observed in normal mice, but did not produce positive cultures.

3) M. alternans mycelium can produce arthrospores in-vivo. After breaking-off, the arthrospores can be visualized as single cells disseminating the disease. Germination of arthrospores would account for the presence of hyphae in distant foci of infection, as observed in histopathological sections.

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