ABSTRACT

INVESTIGATION OF THE VIRULENCE AND PHYSIOLOGY OF SOME STRAINS AND MUTANTS OF BLASTOMYCES DERMATITIDIS, GILCHRIST AND STOKES 1898

By

Samuel Truman Bass

There is very little information on the enzyme activities and virulence of Blastomyces dermatitidus Gilchrist and Stokes 1898. The objectives of this research included the development of less virulent strains of B. dermatitidis, and the study of alkaline and acid phosphatase in relation to virulence. Other factors including lipids in relation to virulence and production of a toxin were investigated.

Mutants of B. dermatitidis St. Joseph strain were developed by ultraviolet irradiation from yeast phase cells. Twenty-six of the 45 isolates were selected for study of nutritional requirements, enzyme production, and virulence in mice. The mutants M-25, M-26, M-37 were found to be up to 100 times less virulent than the parent St. Joseph strain.

The basal salts medium of Gilardi and Laffer was used for amino acid and vitamin studies. When asparagine was a supplement nitrogen source in the medium, the St. Joseph and

Ga-1 strains had good growth in the yeast phase only. When proline was added, both the mycelial and yeast phase showed increased amount of growth. In order to maintain viable cultures in the yeast phase for more than 5 to 8 days, asparagine was necessary in the medium of Gilardi and Laffer. Glycine, tyrosine, and proline could not be substituted for asparagine. The vitamins, biotin and thramine were not required for the growth of B. dermatitidis.

In the study of the alkaline and acid phasphatase activity, an enriched medium, CYPG, consisting of casein, yeast extract, peptone and glucose, was used. The extra cellulose alkaline phosphastase activity was generally higher than the acid phosphatase in the liquid medium at 37 C and at 24 C. The levels of the extracellular alkaline and acid phosphatase activities remained lower at 37 C than at 24 C over a 30 day period. The intracellular alkaline and acid phosphatases were usually much higher than the extracellular enzymes for both phases. Both enzymes were not easily removed from the cell membrane and were found not to be particle bound. There seemed to be no correlation in the level of alkaline or acid phosphatase activity and virulence in mice.

The lipid content in yeast cells of strain of B. dermatitidis selected varied with no apparent correlation to virulence in mice. When tripsin treated yeast cells at 40 mg/mouse were injected into mice, the cells were more virulent than untreated cells.

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By

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To my wife

and

children

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INTRODUCTION

The systemic fungus Blastomyces dermatitidis (Gilchrist and Stokes, 1898) forms granulomalous lesions in many parts of the body, with most of the fatal cases involving the pulmonary region. The organism was first demonstrated by Gilchrist and Stokes (1896), and until about 1950 it was extremely difficult to treat. In 1959 Baum and Swartz reported that blastomycosis is almost a conquered disease with the discovery of two antifungal agents, amphotericin B and stilbamidine and its structural antilogs.

The nutritional requirements, including vitamins and amino acid requirements, and factors influencing dimorphism, have been studied by a number of investigators. However, very little information is available on the enzyme activities and virulence of B. dermatitidis. Urease and uricase activities were reported in cultures and extracts of the fungus (Taylor and Johnson, 1962; Taylor, 1962) and alkaline and acid phosphatase activities were reported by Beneke, Wilson and Rogers (1969).

Denton and DiSalvo (1963) have shown a possible connection between the amount of lipids and the virulence of different strains of the organism. This is the only report of a specific material being associated with the virulence

of the disease blastomycosis.

The objectives of this research include the development of mutants from Blastomyces dermatitidis by irradiation and selection of variant strains for further investigation. Any changes in virulence of the mutants will be determined in laboratory animals. Less virulent strains or avirulent strains will be selected for future research concerned with investigations of a viable vaccine. The desired type mutant was an avirulent strain for use in viable vaccine research in animals. The growth requirements of mutants will be compared with the nonmutant strain of B. dermatitidis to determine if changes have occurred. A comparison will be made between virulence of the mutants, and strains of B. dermatitidis, and variation in the amount of alkaline and acid phosphatase activities or other enzymes in the mycelial and yeast phases. Other factors, including lipids in relation to virulence and production of a toxin, will be investigated.

REVIEW OF LITERATURE

Classification of Organism

Blastomycosis is a fungus disease of humans and animals caused by Blastomyces dermatitidis, Gilchrist and Stokes (1898). The organism has been renamed a number of times, even though the initial paper by Gilchrist and Stokes (1898) gave a good description of the morphology of B. dermatitidis as an organism with yeast cells 10 to 20 μ in diameter at 37 C, as well as having mycelium in cultures at room temperature. In 1901 Ricketts reviewed blastomycosis and concluded that all the organisms that Busse (1894), Curtis (1896) and Gilchrist (1898) had observed were the same, and renamed the etiologic agent Oidium dermatitidis. This position was supported by Bassoe (1906), when he pointed out that the cases from Europe, California and Chicago were caused by the same organism. Another author, Vuillemin in 1901 called it Cryptococcus gilchristi.

In 1907, Hamburger compared four cases reported by Bassoe (1906), one by Iron and Graham (1906) and two by Christensen and Hektoen (1906) and considered these were the same as those described by Gilchrist and Stokes (1898), and he called the organisms from these cases B. dermatitidis.

Ludvig and Hekloen (1907) suggested that certain differences could be observed between the fungus from California and the one found in Chicago, and should be called Oidium dermatitidis (Ricketts, 1901). The literature after 1907 contains numerous papers on blastomycosis caused by a yeast-like organism. deBeruman and Gougerot (1909) renamed this organism Zymonema gilchristi. Pollecci and Nannizzi (1927) called the organism that caused blastomycosis Glenospora gammeli. In 1928 Castellani changed the classification of these organisms. He formed a new genus, Blastomycoides, with three species, B. dermatitidis, B. immitis and B. tulanensis. Later B. tulanensis was considered a synonym of B. dermatitidis. Another renaming occurred in 1929 when Dodge and Ayers called the organism Endomyces capsulatum. Agostini (1932) renamed the organism Monosporum tulanensis, while Castellani (1933) called the organism Glenospora brevis.

A review of the etiologic factors in "blastomycosis" was made by Moore in 1933. The term "blastomycosis" to include not only Gilchrist disease but a number of species of the genera Saccharomyces, Monilia, Cryptococcus, Endomyces, Sporotrichum and others. Moore (1933) renamed the organism Endomyces dermatitidis because he thought an ascus was present. An earlier report of an ascus as the perfect stage of the organism that causes blastomycosis was made by Whitman (1913). Dodge (1935) renamed the organism Zymones dermatitidis and Z. capsuletum.

In 1939 Martin and Smith classified the disease into two clinical types of infection; 1) cutaneous blastomycosis, which proceeds as a chronic or subacute ulcerating process, and 2) systemic blastomycosis, a highly fatal disease, characterized by pulmonary infections with wide-spread distribution of lesions in the subcutaneous tissues, bones, joints, central nervous system and internal organs. These authors considered that the same organism causes both types of infections.

In 1942 Conant and Howell made a comparative study of eight cultures from South American blastomycosis and seven cultures from North American blastomycosis. They found these fungi to be sufficiently similar and proposed the same genus Blastomyces for both. The two species were recognized as B. brasiliensis, the etiologic agent of South American blastomycosis, and B. dermatitidis, the etiologic agent of North American blastomycosis.

In the discussion in a report by Conant and Howell (1942), Moore and Weidman suggested that the two species should be in two separate genera. Hence the North American blastomycosis is currently named Blastomyces dermatitidis, Gilchrist and Stokes (1898) and South American blastomycosis is known as Paracoccidioides brasiliensis, (Splendore) Almeida, 1930.

An excellent key was devised by Gordon (1952) to distinguish between the many fungi which caused diseases of human beings and animals. The key for North American

blastomycosis reads:

> GG_1 Double-contoured spherical cells, 8-15 μ in diameter: buds, when present, single and often separated from mother cell by a broad septum. May be confused with single-budding or non-budding forms of B. (or P.) brasiliensis, immature spherules of Coccidioides immitis, or rounded arthrospores of Geotrichum candidum. Occurs only on the North American continent (Blastomyces dermatitidis).

Carmichael (1962) transferred B. dermatitidis to Crysosporium dermatitidis on the basis of the aleurosporic nature of the conidia. However, this has not been generally recognized as the genus is too heterogeneous for a useful generic concept.

The classification was clarified when McDonough and Lewis (1967) reported the perfect stage of Blastomyces dermatitidis. The cleistothecia produced eight spored asci. The monoascospored culture developed hyphae and conidia typical of B. dermatitidis. These cultures were injected into mice which became infected with the disease blastomycosis. These authors concluded that comparison of this fungus with descriptions of members of the Gymnoascaceae indicates that the organism belongs to this family and to a new genus.

In 1968 McDonough and Lewis described the perfect stage of B. dermatitidis, in the family Gymnoascaceae, as Ajellomyces dermatitidis. These authors conclude that only one species is present in the United States. The six isolates from Africa (Ajello, 1967) in preliminary crosses appear to be the same species as the isolates from the United States.

McDonough (1968) suggests that in a study by Moore (1933) that the illustrated "asci" were in reality ergastic globules. Kwon-Chung (1970) along with McDonough found that ascospores separated randomly in Ajellomyces dermatitidis could be used for genetic studies.

Cases of Blastomycosis

Since Gilchrist first reported the disease blastomycosis in man in 1896 until today, the disease has been a very serious infection; in many cases blastomycosis has been terminal. A number of early reports on cases of blastomycosis are in the literature: Ricketts (1901), Stober (1914), Castellani (1928) and Moore (1933). The first negro with the disease was reported by Gilchrist in 1902, hence the disease is not specific as to the race of the host. A number of recent reviews of cases of blastomycosis have been reported in the literature including those by Duttera and Osterhout (1969), Witorsch and Utz (1968), and Busey (1964).

The disease blastomycosis can express itself in two forms--the first of these is the deep mycotic infection or systemic infection and the second is cutaneous. In 1907 LeCount and Myers in describing a patient said, "The patient was literally eaten up by the disease." They cultured the organism and obtained large yeast cells with buds. This case was a systemic infection.

In 1916 MacLane found 3 cases of generalized blastomycosis, but in addition reported that one of the three was able to ferment saccharose (sucrose), maltose, dextrose, levulose (fructose) and mannite (mannose).

Baker (1942) concluded that human blastomycosis could be interpreted as being primarily pyrogenic, with prominence of the polymorphonuclear neutrophils. Some lesions of cases, especially in the systemic group, closely resemble the lesions of tuberculosis.

In the terminal stages of systemic blastomycosis in man the lesions corresponded closely to the experimental disease in mice (Baker, 1942a). Masses of the organisms occurred with necrosis, producing a toxic effect upon the patient. In cutaneous cases, in contrast, organisms were usually moderate in numbers; caseous necrosis was usually absent and the lesion was composed of miliary abscesses, granulation of tissue and hyperplastic epidermis. In cutaneous cases, little toxic effect was observed upon the patients.

Baker (1942b) concluded that from these observations on the nature of toxicity in blastomycosis that therapeutically a fungicide is not desirable in the severe systemic cases, since too much necrotic blastomycotic material is already present.

In 1955 Schwarz and Baum reported that the most frequent infections orginate with pulmonary lesions. Many cases of cutaneous lesions have been reported, but only

three cases of infection by injury have been substantiated. In these cases; doctors obtained the disease while working on the autopsy. A very similar report by Wilson et al. (1955) showed that North American blastomycosis occurs with extreme rarity, with four known cutaneous cases, as a result of direct inoculation of the organism into the skin. The primary cutaneous blastomycosis presents a chancriform syndrome which is entirely different from the usual chronic cutaneous form. There is apparently a strong tendency toward spontaneous recovery in primary cutaneous blastomycosis.

The disease blastomycosis attacks the skin (Montgomery, 1902) and (McCaulay, 1956), the lungs (Schwarz and Baum, 1955), the kidneys (Toepel, 1929), the bones (Toepel,1929), the heart (Baker and Brian, 1937), the brain tissue (Gaspas, 1929) and (McBryde and Thompson, 1933) and many other tissues (LeCount and Myers, 1907) of human beings.

Blastomycosis is also a disease of many types of animals. Chick reported 58 dog cases (1966) in an editorial article. An early reported animal case was in a dog (MacLane, 1916). The disease also was found in a horse (Benbrook et al., 1948), a sea lion in the Chicago Zoo (Williamson, 1959) and a Siamese cat (Easton, 1961) in Canada. The disease in the cat was called a cutaneous infection, while the horse had a mammary gland infection and the sea lion had a deep mycotic infection.

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In a more recent review, Ajello (1967) reported 116 cases of dog blastomycosis, 113 of these cases were from the U.S. and three were from Canada. It was concluded that the canine is the primary animal susceptible to infection by B. dermatitidis.

Geographic Distribution

Blastomyces dermatitidis has been thought to be only able to survive in the North American continent. However in 1964 autochthonous cases of indisputable B. dermatitidis infections were reported by three different groups at the same time, Emmons et al. (1964), reported two cases, a case was reported by Destombes and Drouhet (1964), and a case was reported from the Congo by Gatti et al. (1964). More recent reports have shown wide distribution of this disease in Africa (Campos, 1968).

Outside the United States and Canada occasional cases of blastomyces have been reported in Venezuela (Montemayor, 1954) and Arias (1962) in Mexico.

Chick et al. (1960) reported that 735 cases had occurred within the boundaries of the United States, while Grandbois in 1963 reported on all published and unpublished cases of blastomycosis in Canada and found 117 such cases in the country. These cases were primarily in the provinces of Quebec (71 cases) and Ontario (34 cases), while the remaining 12 cases were in four other provinces, Manitoba

(8 cases), New Brunswick (2 cases) and one case each from Nova Scotia and Saskatchewan.

As to the distribution of the disease in the United States, the states with the highest prevalence of cases either border on the Mississippi River or are situated east of that river. The following states had 15 cases or more reported in 1960: Arkansas, 15; Illinois, 74; Indiana, 18, Iowa, 24; Kentucky, 37; Louisiana, 101; Tennessee, 90; and Wisconsin, 81 (Chick, 1960).

In another review of V. A. Hospitals in the United States (Bussey, 1964), 198 cases, covering the period of time from 1946-1957, the disease was reported to be more prevalent in the Middle Atlantic, South Central, and the Ohio-Mississippi River Valley States. Chick in 1966 reported an additional number of cases for West Virginia (51 cases); this indicates an increase from less than 15 cases in 1960, to 51 cases in 1966; he also reported an increase of 110 cases for Kentucky, and 15 additional cases in the state of Tennessee. The large increase in number of cases in these states may indicate that in the earlier survey (Chick, 1960) many records of cases of blastomycosis were not included, which would in turn indicate that many more cases of blastomycosis may be on hospital records in this country.

Natural Habitat

The natural habitat of B. dermatitidis remains elusive (Ajello, 1967). This is stated despite recent reports of isolation from soils of B. dermatitidis in Georgia (Denton and DiSalvo, 1964) and Kentucky (Denton et al., 1961).

The soil studies of Denton and DiSalvo (1964) found 10 of 356 soil samples positive to B. dermatitidis. The positive sites were in living quarters of man, mule, cattle, chickens and rabbits, which certainly would not narrow down the natural habitat to a specific environment. Other problems were encountered with this work, such as repeated samples for the organism from former positive sites, consistently turned out to be negative.

The stored soil samples lost their ability to produce viable cultures of B. dermatitidis, except in one case, according to McDonough et al. (1961), when the soil was kept at room temperature for 16 months in a screw top bottle. McDonough in other studies (1963, 1965) showed that natural soils would not maintain B. dermatitidis and the yeast phase was lysed. However, if the soil is sterilized before inoculation, the fungus has a much better chance of survival. Other workers (Bakerspigel, 1953; Fennell et al., 1950) in an effort to find a storage media for B. dermatitidis used sterilized soil, and reported similar results. To explain what happens to the organism

in the soil McDonough suggests that some factor in the soil causes lysis (1970), (Friburg, 1970).

Denton and DiSalvo (1968) reported that laboratory animals could become infected with sterilized soils seeded with the saprophytic form of a highly virulent strain of B. dermatitidis. The facts reported by the above workers would indicate that somewhere in nature an ecological niche exists that favors B. dermatitidis.

Dimophorism of Blastomyces dermatitidis

A. Temperature.

The interconversion of mycelial (M) and yeast-like (Y) forms in B. dermatitidis and in P. brasiliensis are characterized as examples of thermal dimorphism (Nickerson and Edwards, 1950), (Salvin, 1949). The phenomena are apparently dependent only on the temperature of incubation of these species. Salvin (1949) pointed out that temperature is the environmental factor controlling the form of growth. He also showed that no amino acid, carbohydrate or growth substance was required for the organism in the yeast phase, but noted the presence of some pseudohyphae in the rich yeast extract and peptone medium. Guidry (1967) reported that the optimum temperature for the yeast-phase growth was 35 C. for 8-12 hours. These results are in agreement with earlier experimenters (Ricketts, 1901; Hamburger, 1907), who reached the same conclusion.

B. Other Factors.

In 1928, Michelson suggested that unfavorable environmental conditions produced by drugs, dyes, bile and incubation temperature caused the organism to revert to yeast stage. He also reported that the yeast-like growth is the resistant form of the organism and the hypha growth is the saprophytic form of the organism. Nickerson (1948a) showed proflavine, cobalt and penicillin promoted Y to M changes, while cysteine prevented the Y to M change.

Weeks (1964) reported the ability for the organism to change to the Y form is enhanced in a cottonseed medium while the pH level and the concentration of the glucose had no effect on the conversion process. The 22 isolates of B. dermatitidis used in his work were converted at a much faster rate than the medium proposed by Kelley (1939).

A recent report by Collins and Edwards (1970) is of interest since the filamentous form of B. dermatitidis was first demonstrated in tissue, while in earlier reports of mycelial forms were not well documented. In earlier reports, the time factor may not have been considered in preparation of sections from tissues, but in this current report only the tissue of animals that were dead for less than 10 minutes was used. Hence there was no chance for the organism to revert to the mycelial form after the mouse had died.

Nutrition of the Organism

A. Introduction.

The nutrition of B. dermatitidis has been reviewed recently by Gilardi (1965), and earlier by Rickett (1901) and Stober (1914). The areas of nutrition that have been treated with the most effort are: media to be used to isolate the organism (Ricketts, 1901; Stober, 1914; Kelly, 1939), media with basal requirements for growth, media to determine carbon and nitrogen sources and media to determine differences in requirements for the two growth forms of the organism at 24 and 37 C (Weeks, 1964; Gilardi, 1965).

B. Vitamin Requirements for Growth.

The recent literature is somewhat confusing in that Halliday and McCoy (1955) have reported biotin is required for growth. They also reported that the only substance that could replace biotin was D-L-desthiobiotin which gave one-half activity of biotin.

The investigators who reported no vitamin requirements were needed are Levine and Ordal (1946), Salvin (1949), Gilardi and Laffer (1962), and Nickerson and Edwards (1950), who may have had biotin in the cotton plugs or in the tubes. The addition to the medium of vitamins was not stimulatory for growth of the organism (Salvin, 1949; Gilardi, 1962). The answers these latter investigators gave for the results

of Halliday and McCoy (1955) were that Salvin (1949) used sealed tubes and extensively washed agar in his experiments and Gilardi and Laffer (1962) used liquid culture medium with aluminum caps.

C. Other Nutrients.

The mycelial form of B. dermatitidis does not require any amino acid for growth (Levine, 1946; Nickerson, 1950). The yeast-phase of the organism grows without the addition of organic nitrogen or vitamins (Gilardi and Laffer, 1962). Levine and Ordal (1946), and Gilari and Laffer (1962) indicate that amino acids are not required, but are stimulatory for growth of both the yeast and mycelial forms of the organism.

Gilardi and Laffer (1962, 1961) showed the organism assimilated 67 carbon and 37 nitrogen substrates as the sole source in liquid cultures. These included hexose, amino acids and some of the Kreb cycle acids as the sole carbon sources. The nitrogen source included ammonium salts; aliphatic amino acids; imino acids, proline and hydroxyproline; acidic amino acids, aspartic and glutamic; basic amino acids, histidine, arginine and lysine, and other amino acid relatives.

Dzawachiszwili, Laudau, Newcomer and Plunkett (1964) showed that sea water reduced the growth one-third when compared to distilled water or 0.85% NaCl for B. dermatitidis. However if 1.7% NaCl is used growth inhibition of

50% is observed, at 3.4% NaCl inhibition is about 80-85%, and at 6.8% NaCl inhibition is 100%. However, Smith and Furcolow (1969), in studies with NaCl concentrations on the growth of B. dermatitidis, reported an increase of almost 2-fold in growth with 1-3% NaCl. The concentration of 5-7% restricted the growth of the organism slightly.

In another report, Smith and Furcolow (1964) showed B. dermatitidis developed the largest number of conidia with a small number of hyphal elements on starling manure infusion medium. The repeated transfer of B. dermatitidis on starling manure infusion resulted in an increase in the number of total particles, the percentage of microconidia and the total viability.

D. Basal Media for B. dermatididis.

The simplest media used in the literature are the ones given by Stewart and Meyers (1938) where ammonium acetate or acetamide are the sole source of carbon and nitrogen for the organism in the yeast phase. These authors reported that the changes in hydrogen ion concentration (pH) appear to be independent of sugar consumption. The basal media reported by Levine and Ordal in 1946, Salvin in 1949 and Gilardi and Laffer in 1962, used glucose as the sole carbon source and ammonium sulfate as the sole nitrogen source. Salvin (1949) suggested that a stimulatory effect can be seen with amino acids.

E. Special Media.

The media of Salvin (1949) was used to study morphology of the dimorphic B. dermatitidis. He concluded that the organism changed from the yeast to mycelial phase solely on the basis of temperature change from 35 to 24 C. Guidry (1967) studied the optimum temperature for the yeast phase and found to be at 35 C, or the lowest temperature to maintain the yeast phase. Weeks (1946) used a special type of medium to get B. dermatitidis to change from the mycelium to the yeast phase. His cottonseed medium showed a much more rapid change over three other media which had been used to change the mycelium to the yeast phase of B. dermatitidis.

Marwin (1956, 1959) found four non-ionic surface-active substances that accelerate growth to nine different human pathogens, and B. dermatitidis was one of these.

Another type of nutritional study was reported by Rosenthal (1964) which would allow one to distinguish B. dermatitidis from 27 other fungi by the nutrient it was able to hydrolyze. He used the following five substances: tyrosine, casein, urea, gelatin and starch. B. dermatitidis was able to hydrolyze tyrosine, casein and urea and was not able to hydrolyze gelatin or starch.

Metabolism

A. Lipids Isolated from B. dermatitidis.

Peck and Hauser (1938) reported that the lipids of the organism contained two separable types; about two-thirds of the lipid was acetone soluble fat and the other one-third acetone insoluble phosphatide. These workers demonstrated that the phosphatide contained glycerol phosphoric acid, choline and ethanolamine. In the acetone soluble fraction they identified glycerol, ergosterol, and palmitic, oleic, and linoleic acids.

Two years later the same workers, Peck and Hauser (1940) found that their extraction technique was not removing all the lipid; after exhaustively extracting with alcohol, ether, and chloroform the solids contained 5.7% of the lipid. Peck (1942), using the lipid from B. dermatitidis, made soap and tested it against the inhibition of the enzyme trypsin.

Baker (1942a) reported that after repeatedly injecting phosphatide intraperitoneally into mice, cells of the monocytic series responded, but this fraction is not responsible for the polymorphonuclear response and the necrotizing effect related to the living organism.

Al-Doory and Larsh (1962) showed that the cell extract of B. dermatitidis, as well as other fungi, must be digested and reextracted in order to obtain total lipids. The total lipid for B. dermatitidis was 16.9% for the mycelium at 15 days and 40.59% for the yeast at 4 days.

DiSalvo and Denton (1963) measured the lipid content of four strains of the organism that had differences in virulence, and suggested that the lipid fraction in addition to causing granulomatous reactions might also be related to the virulence of various strains of the organism for mice and, by inference, for man.

B. Polysaccharides.

Peck and Hauser (1938) isolated polysaccharides from cell autolyzates of B. dermatitidis. They precipitated the polysaccharides with ethanol, removed proteins from the preparation with chloroform, NH_4OH or chloroacetic acid. The amount of the polysaccharide was about 1% of the total dried cells. They were able to show that patients with blastomycosis gave positive allergic reactions to the polysaccharides from the organism.

Baker (1942a), using the polysaccharide of Peck et al. (1940) in rabbits, found that a single intraperitoneal injection of the blastomycetic polysaccharide produced, in the first few hours, sterile peritonitis, retrosternal lymphadenitis and remarkable changes in the blood stream consisting of leukopenia, lymphopenia and increase in the number of immature amphophils. A similar change was observed by Sabin et al. (1938) with polysaccharides from tubercle bacilli and pneumococci.

C. Toxins.

A number of saprophytic fungi have been found to produce toxic reactions (Gortner and Blakelee, 1914; Abel and Ford, 1907) when injected into animals. Salvin et al. (1952) was able to demonstrate that Candida albicans has a toxic effect in mice.

Baker (1942a) showed that repeated intraperitoneal injections of heat-killed B. dermatitidis were toxic for mice, and often lethal. This is thought to explain the final lethal effect in the experimental disease, in which masses of organisms and intermingled reacting cells become necrotic, and probably permitted the absorption of substances such as those associated with the suspensions of the heat-killed organism. He injected 0.1 mg into mice every other day for 8 weeks with no apparent effect. When 10 mg was given every other day, the animals appeared ill after each injection; most animals died 5 to 11 days, apparently of a toxic effect of the suspension. The body cavity, when examined, showed the peritoneum was lined with a friably, yellow layer, which was composed mainly of killed B. dermatitidis.

An endotoxin has been demonstrated by Salvin (1952) with acetone dried cells. The addition of 2 mg of tubercle bacilli along with the 1.7 mg of acetone dried cells gave an LD_{50} in mice for this system in 48 hours for B. dermatitidis. He found that the components from 80 mg of acetone

dried cells, injected intraperitoneally, caused death in about 80% of the mice in 48 hours. Taylor (1964) found a similar toxin released with trypsin treatment of B. dermatitidis cells as well as when pre-treated with 1% HCl solution.

D. Enzymes

Rosenthal (1964) used a media with a specific substrate to assist in identification of specific pathogenic fungi. Today, this technique is used in a routine way to distinguish between two morphologically similar dermatophytes, Trichophyton mentagrophytes and T. Rubrum. T. mentagrophytes hydrolyzed urea while T. rubrum will not hydrolyze this substrate (Philpot, 1967).

Urease was shown to be a specific substrate enzyme in B. dermatitidis by Taylor and Johnson (1962). These workers showed the mycelial and yeast phase exhibited a constitutive urease activity, both in growing cultures containing urea as the sole nitrogen source, and in cell extracts. The optimum production of the enzyme occurred at a pH of 7.0 with urea as the sole nitrogen supply.

Taylor (1962) found an enzyme system in one form of the organism but not in the other phase. The mycelial phase utilizes uric acid but not the yeast phase. At high pH, 9.0 this activity is inhibited, however the organism grows well and uses uric acid at a pH of 6.5, 7.0, and 8.0 at 25 C. Taylor could not find evidence of uric acid being

used by the yeast-phase of the organism at any hydrogen ion concentration tested.

The extracellular enzyme of B. dermatitidis has been examined by Beneke, Wilson and Rogers (1969). These authors found acid and alkaline phosphatases in the culture medium extracts. They noted that with time an increase in activity could be shown. A difference in the amount of these phosphatases in the two dimorphic phases was noted in the culture extracts.

Other enzymes that showed no activity in the culture medium when tested on substrates by Beneke, Wilson and Rogers (1969), were: β-glucosidase, α- and β-galactosidase, N-acetylglucosamidase, and acetate, butyrate, palmitate, stearate and laurylate esterases.

Rippon and Varadi (1968) tested three strains of B. dermatitidis and found no activity for the enzyme elastase, while a number of other fungi did give a positive elastase activity.

Rippon and Lorincz (1964) determined the collagenase activity for 37 different pathogenic fungi, with 35 of these negative and the following two positive: T. schoenleinii and Streptomyces madurae, Blastomyces dermatitidis was one of the 35 organisms that gave a negative collagenase activity.

Roy et al. (1970) studied the polymorphic enzyme malate dehydrogenase which is found in many fungi. These workers studied the yeast and mycelial phase of B. dermatitidis.

The method used in these studies involved separation of the mitochondria from the cytoplasm by differential centrifugation. Gel electrophoresis was used to show the many forms of the enzyme. Five bands were present in the cytoplasmic fraction, and only two in the mitochondrial fraction of the yeast phase of the organism.

E. Other Biochemical Properties.

Stewart and Meyer (1938) have shown that B. dermatitidis and Coccidioides immitis differ from the metabolism of bacteria, in that the production and assimilation of ammonia is favored in these organisms even in the presence of glucose. This work was supported later by Bernheim (1942) who demonstrated that ammonia was produced from the natural and non-natural isomer of the amino acids. These amino acids were not deaminated in the process of being metabolized.

Bernheim (1942) showed that oxygen uptake increased in the presence of amino acids. He also demonstrated that lower fatty acids are oxidized, but higher fatty acids inhibit the control respiration and the oxidation of added substrates. He found that cyanide inhibits the oxidation of all added substrates, but has comparatively little effect on the control of respiration possibly because it fails to penetrate into the cells. Nickerson and Edwards (1950) showed that the yeast phase consumes 5 to 6 times more oxygen per unit dry weight than does the mycelial phase. The

yeast phase shows exogenous oxidation of acetate and glucose while the mycelial phase shows no exogenous oxidation. Levine and Novak (1950) studied the respiration of the yeast phase of the organism. They found the carbohydrates, glucose, mannose and xylose, stimulated respiration, while mannitol, glactatol and sorbitol stimulated oxygen uptake. The fatty acids, increasing in size through caprylic acid, stimulated oxygen uptake, larger fatty acids inhibited respiration. The respiration was not affected when KI was added. Tyrothricin inhibits endogenous respiration and 1/600 M sodium azide increases the endogenous respiration.

Taylor (1961) demonstrated that the dimorphic forms of B. dermatitidis had no difference in total DNA. He found that only RNA in the mycelial phase increases at a slightly slower rate, than in the yeast phase. This author found the trichloroacetic acid (TCA) nitrogen concentration to be much higher in the yeast phase than in the mycelial phase.

Brunelli (1963) tried to associate the amino acids with pathogenicity, but no correlation was found. He did, however, show that B. dermatitidis could be characterized by the absence of proline and the uniform distribution of the other amino acids.

The gas ethylene was shown by Nickerson (1948b) to be released from the cultures of B. dermatitidis.

F. Cell Wall and Ultrastructure.

The work of Venezuelian workers Carbonell and Kanetsuna (1968), and Kanetsuna and Carbonell (1969) correlate structure and cell composition of P. brasiliensis and B. dermatitidis. The isolated or intact cell walls were studied by electron microscopy by use of enzymes and solvents. Both fungi showed fibrils measuring 100 A at the external surface and an inner granular layer. Sections showed an external dense layer and an inner stratum devoid of a distinct fibrillar structure. Glucan and chitin were the main components of the cell wall, in addition to small amounts of lipids and proteins. The data suggest that the external layer is composed mainly of glucans. The ultrastructure of the organism has also been described by Brown and Edwards (1968), and Garrison, Lane and Field (1970).

Animal Inoculation

Many of the early workers infected laboratory animals as a confirmatory diagnosis for blastomycosis (Ricketts, 1901; Stober, 1914; Hamburger, 1907). Several different kinds of animals were used, but mainly rats, mice, guinea pigs, and dogs were used for intraperitoneal injection of the organism.

Baker (1938) compared the effect of yeast-phase and mycelial phase in mice. He was unable to show one form of

the organism to be more infective than the other. He noted that regardless of the initial form of the organism, the only form found from the infected animals was the yeast-phase of B. dermatitidis. Baker (1942a) reported that mice were the best laboratory animals for the study of blastomycosis in animals. Smith et al., (1966) using viable mycelial particles (144,000) found that hamsters were the most susceptible animals to infections induced by intraperitoneally injecting the organism.

Baker (1939) found that the repeated passage of an organism into a mouse did not increase the virulence nor did it change the cultural characteristic of the organism. Landy et al. (1970) reported that a difference in susceptibility exists because of sex in hamsters, and that the male animals are more susceptible than the female animals.

Landay et al. (1968) showed that hamsters could be infected by three routes: intramuscular, subcutaneous and intraperitoneally. Heilman (1947), using mice, found that by intravenous injections, the period of time to death could be related to the number of organisms. When 1.84×10^5 cells were used of one strain, death came to the animal in 7-8 days.

Conti-Diaz et al. (1970a, 1970b, 1969) found that male hamsters were quite susceptible to Blastomyces dermatitidis if inoculations were intratesticular. A hundred organisms or less for intratesticular inoculation resulted, at the end of a 35-38 day period, in positive cultures of the organism from the animals.

Salfelder (1965) was able to infect hamsters with cutaneous infections. The disease in the hamsters seems to be comparable to the cutaneous disease of man. The organism was not disseminated throughout the body as was true with the intratesticular inoculation reported by Conti-Diaz et al. (1970a, 1970b).

Miner et al. (1969) attempted to lower the error in inoculation of mice. They observed such variables as the size of inoculation needle, site of penetration, individual who inoculates, angle of injection and the speed of injection. None of these factors seemed to improve the system for test of virulence. Denton and DiSalve (1968) treated soils with conidia and allowed the animal only a short time in contact with this soil, then they were put into a new cage. These animals developed a high percentage of blastomycosis. This technique may aid in finding additional soils containing the organism.

MATERIALS AND METHODS

Blastomyces dermatitidis Cultures

Eleven isolates of the organism Blastomyces dermatitidis were obtained from three different laboratories. These isolates were from man, dog and sea lion. The organisms were grown on Sabouraud's glucose agar at 24 C, and on the medium known as CYPG medium (see Media) at 24 or 37 C. All inoculations were made from the yeast phase of the organism for cultures at 24 and 37 C, as well as for mice inoculation. Transfers were made under a transfer hood for cultures as a safety precaution.

The isolates used in these experiments were obtained from the following laboratories:

A. The laboratory of Dr. E. S. Beneke, Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan.

1. St. Joseph Strain--isolated from a patient at St. Joseph Hospital, Ann Arbor, Michigan.

2. Van Camp Strain--isolated from a dog, in the Veterinary Clinic, Michigan State University, East Lansing, Michigan.

3. Oklahoma Strain--isolated from a patient in the Oklahoma Medical Center, The University of Oklahoma, Oklahoma City, Oklahoma.

4. Duke Strain--isolated from a patient in Duke University, Durham, North Carolina. This isolate had been in culture for more than 20 years.

B. Cultures obtained from Dr. J. F. Denton, Department of Microbiology, Medical College of Georgia, Augusta, Georgia.

1. Ga-1--isolated from a human case of chronic cutaneous blastomycosis at the Talmadge Hospital on December 6, 1958.

2. B-130--isolated from a human patient with a chronic ulcer of the leg at Chester, Pennsylvania.

3. S-182--isolated from lymph nodes of a seven year old male dog at Cincinnati, Ohio.

4. SL-1--isolated from a northern sea lion that died in the Chicago Zoological Park.

5. KL-1--isolated in April 1960 from a soil sample collected near Lexington, Kentucky. First soil isolate.

C. Cultures received from Dr. E. S. McDonough, Department of Biology, Marquette University, Milwaukee, Wisconsin.

1. ATCC 18187 (A) Dr. McDonough number B-784 (A).

2. ATCC 18188 (a) Dr. McDonough number B-788 (a).

Media

A. CYPG Medium.

The medium, known as CYPG was used by Beneke, Wilson and Rogers (1969) as a nutrient rich medium. It contains 4 g each, yeast extract, peptone and casino amino acid and 10 g of glucose per liter. Cultures of B. dermatitidis were grown at 24 and 37 C for enzyme studies both on solid and liquid media.

B. BHI Medium.

Difco brain heart infusion medium, with or without agar was used for growth of yeast phase of strains of B. dermatitidis.

C. Basal Media.

These media were used to study the nutritional requirements of the mutant strain.

1. The medium described by Gilardi and Laffer (1962).

Basal Medium = BM

Glucose	10.0 g
Ammonium sulfate	5.0 g
KH_2PO_4	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2 \cdot 2H_2O$	0.1 g
NaCl	0.1 g

One of the many variations of this medium is the BM with asparagine at 3 g per liter = aBM. Other amino acids were added to this medium at 1 g per liter, also biotin,

thiamine and inositol, which had been implicated in B. dermatitidis nutrition, were added to this medium.

2. Levine and Ordal Medium (1946).

Glucose	10.0 g
Ammonium Sulfate	5.0 g
K_2HPO_4	5.0 g
KH_2PO_4	5.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
NaCl	0.1 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$MgSO_4 \cdot 2H_2O$	0.064 g
Water	1,000.0 ml

This medium with high buffering capacity was used to see if the pH of medium could be better controlled during the growing period of the organism.

3. The Medium of Salvin (1949).

Glucose	10.0 g
Ammonium sulfate	5.0 g
Na_2HPO_4	5.0 g
NaCl	4.0 g
KCl	1.0 g
$MgSO_4 \cdot 7H_2O$	0.5 g
$CaCl_2$	0.025 g
$FeCl_3$	0.010 g
Water	1,000.0 ml

This medium was also used to see if it would support the growth of B. dermatitidis in both phases.

Basic Requirements for Growth

A. Basal Medium.

The basal medium reported by Gilardi and Laffer (1962) was used as a basis in a number of nutrient experiments. The medium consisting of inorganic salts plus glucose (BM) and 300 mg/100 ml of asparagine (aBM) was used to determine

the requirements for growth of certain mutants. This medium was supplemented with amino acids and with vitamins in various combinations, to check growth requirements of the mutants at 37 and 24 C.

B. Basal Media Plus Amino Acids.

The basic medium (BM) was supplemented with 100 mg of a single amino acid, or a combination of up to 9 amino acids, at 100 mg each/100 ml. The amino acids, which in preliminary experiments had shown some possibility of supporting the growth of the organism, were used in this experiment. The yeast phase of the inoculum was started at 37 C on the BM, and after six days was used to inoculate the flasks for incubation at 24 C. The amino acids used in this experiment were creatine, valine, isoleucine, proline, cysteine, hydroxyproline, tyrosine, histidine and serine, which were used individually or in combination.

C. Basal Medium Plus Asparagine Plus Supplements.

The basal medium plus asparagine had other amino acids and nutrients added in some cases. These were 0.3% glycine and 0.3% tyrosine, 10 µg/liter biotin and 100 µg/liter of thiamine, and inositol (10 mg/liter). These cultures were grown at 24 C. At 37 C the cultures appeared to grow no better than the controls without added amino acid and supplements.

Measurement of Changes with the Organism

A. Growth.

Three methods were used to determine rates or amount of growth (Oginsky and Umbreit, 1959).

1. Dry Weight.

A filter pad was dried for at least 8 hours in an oven at 80-90 C, cooled and weighed. The organism was filtered onto the filter pad, washed 3 or 4 times with distilled water, dried at least 12 hours and the difference in weight was the dry weight of the organism. All samples were sterilized before discarding.

2. Wet Weight.

In this method the laboratory vacuum line was used as a source of constant vacuum suction, the organism was filtered on regular filter paper, washed in water, the water coming through the filter dripped into the suction flask. In order to maintain a constant water content these drips were allowed to continue until the time between drops was greater than 30 seconds. The organism was then removed from the filter pad and weighed immediately. This procedure allowed the cells to be used in other experiments where the active proteins were to be studied. All materials were sterilized before discarding.

3. Optical Density Measurements.

Several investigators have used optical density (Guidry, 1967), DiSalvo and Denton (1963), measurements as a method to estimate the number of B. dermatitidis cells based on turbidity. The wave length used is quite varied, for instance Holiday and McCoy (1959) used 625 mμ, as did Gilardi and Laffer (1962), Guidry (1967) used 500 mμ and 550 mμ was used by DiSalvo and Denton (1963). In this work the wave length at 550 mμ was used to measure turbidity.

B. Hemacytometer Bright Line Counting Chamber.

A drop of the organism is placed on the hemacytometer, and spread out over the area by the addition of a cover slip. The number of cells in each of the sixteen small squares are counted and totaled in order to calculate the number of organisms in a large square. Calculations are made by multiplying the number of organisms in this large square by 25, then the product by 10^4 to find the number of organisms per ml.

C. Viability of the Organism.

1. The number of viable cells in the inoculum from stock cultures were plated on mycosel agar (Beneke and Rogers, 1970). When the colonies developed, counts were made to determine the number of viable cells that were present in the original inoculum.

2. Study of Number of Viable Cells on Varied Media at 37 C.

Using the BM and aBM media, the following additions were made: 100 mg/100 ml each of glycine and tyrosine; 100 mg/100 ml each of proline and glycine; 100 mg/100 ml of proline and no additions. The growth of the organism was checked at 5, 8, 12, and 24 days, using the optical density at 550 mμ as an index of growth, to establish a growth curve. The viable cell counts were made at 5, 8, 12, and 24 days.

In another experiment the number of viable cells in a culture at different times were examined. Four specific media were used: CYPG, BM, BM + proline, and aBM + proline. The optical density of the cell suspension was measured at 550 mμ and the viable cells were determined by the Janus Green method (Reca and Campbell, 1967). These two cell functions were measured at 2, 4, 6, 9, 12, and 15 days. On the 15th day other determinations were made, such as pH of supernatant fluid, dry weight, and acid and alkaline phosphatase.

3. Vital Stain Method.

The vital stain was prepared using 20 mg of Janus Green B per 100 ml (0.02%). A 0.1 ml cell suspension or a dilution from the culture was put into a test tube. The 0.1 ml of Janus Green B dye solution was added to the cell suspension and allowed to stand at room temperature for 10 min. A drop of the cell suspension was placed on the hemocytometer for

differentiation of viable and non-viable cells under the microscope. The viable cells do not stain while the non-viable cells stain a purple or dark blue. The percent viability can be determined on the basis of the number of stained cells.

D. Lipid Determination.

The lipid concentration was determined by the methods of DiSalvo and Denton (1963) and Al-Doory and Larch (1962).

The method of DiSalvo and Denton (1963) extracted fats with chloroform-methanol (2:1) as the solvent. The temperature was kept at the boiling point of the solvent in a 250 ml soxhlet apparatus for 48 hours. The Al-Doory and Larch method (1962) gave an additional acid treatment to the cells that were being extracted. These authors called the first extractable lipids the free lipids, and after acid treatment the lipids were called the bound lipids. The method of Al-Doory and Larch (1962) was used to determine the phospholipid. These fats were insoluble in warm acetone.

Mutants of Blastomyces dermatitidis

A. Technique used to Form Mutants

Ultraviolet (UV) light has been used by a number of investigators, Emmons and Hollaender (1939), Fincham and Day (1965), to produce mutation of fungi. The mycelium and

the yeast forms are both affected by UV light, Fincham and Day (1965). The UV lamp (2527 A) was set up in the transfer hood 12 inches above the table top. The St. Joseph strain, B. dermatitidis, was used in this experiment. The organism was cultured on CYPG agar 10 days at 37 C, harvested and washed with sterilized saline solution. The yeast cells were counted with a hemocytometer and adjusted with saline solution to 1.6×10^8 cells/ml. Thirty ml of cell suspension were placed on the bottom of a flat Petri dish which was placed on the table directly under the UV lamp. A 0.1 ml sample was withdrawn from the dish at 2, 4, 6, 8, 10, 15, 30 and 60 minutes and placed in 10 ml of distilled water (1:100 dilution). This dilution was diluted to 1:10,000 dilutions and 1 and 0.1 ml sample of each dilution (1:100 and 1:10,000) was placed into a Petri dish in which they were mixed into the CYPG and BHI media. The Petri dishes were placed in a 37 C incubation oven for two weeks.

After two weeks, 45 cultures were selected for isolation onto CYPG slants from plates of fungi exposed for different times as shown in Table I of the Results. Two weeks later these isolates were examined for variation in cultural characteristics as well as for characteristic cell structures under the microscope. The data on the cultures are tabulated in Table I of the Results. Thirteen cultures were unable to grow on the CYPG medium, two other cultures failed to grow in the next transfer, and the remainder were contaminated. The remaining twenty-six cultures were used in the later experiments.

B. Morphological Study of Mutants at 24 and 37 C.

The cultures of all the UV mutants were placed on BHI agar and incubated at 37 C. The colonies were observed for morphological changes.

The mycelial phase of these same mutants was studied in culture on Sabouraud's agar and on Gilardi and Laffer (1962) basal agar medium at 24 C culture temperature for morphological changes.

C. Virulence of UV Mutants in Mice.

The mutants were tested for virulence in mice. The intraperitoneal injection route was used for inoculation of the mice, with the number of cells injected into the animals ranging from 10^4 to 10^8 cells. The number of viable cells were determined by plating techniques, and counting the number of cells with a haemocytometer (Reca and Campbell, 1967).

Gel Electrophoresis

The preliminary gel electrophoresis of proteins was determined using a modification of the method of Davis (1951). In these experiments the 300-400 μg of soluble proteins to be electrophoresed were not put into a sample gel, but were put into 0.2 ml of buffer or into 40% sucrose solution to aid in applying samples onto the gels. The conditions used on the power supply were 5 milliamperes per gel in the

electrophoresis. The tubes used to make the gels were 8 mm in diameter and 100 mm in length. The separation gel was 65 mm in length. The gels were stained by either Swartz black (Buffalo Black) or commassie blue (Weber and Osborn, 1969). The commassie blue was the more sensitive of the two stains.

Enzyme Studies

A. Acid and Alkaline Phosphatase.

The assay for phosphatases was made using the method described by Beneke, Wilson and Rogers (1969). The samples were taken from near the colony with a 5 mm cork cutter, put into 1 ml of substrate p-nitrophenol phosphate at pH of 5.0 in acetate buffer (0.1 M) for acid phosphatase and at pH of 8.5 in Tris buffer (0.1 M) for the alkaline phosphatase. Some of the filtrates of the mutants with the most active phosphatases were assayed after growth in liquid CYPG medium.

B. Enzyme Rate Measurements.

1. Acid Phosphatase.

The general phosphatase substrate p-nitrophenol phosphate was used to determine the acid phosphatase activity according to the method of Garen and Levinthal (1960). The method was modified slightly for temperature of incubation to 30 C, and a unit of activity is defined as the number of

μmoles of p-nitrophenol released per hour at 30 C. The molar absorbancy index for p-nitrophenol in Tris at pH's higher than 8.5 is 1.62×10^4. The acid phosphatase substrate was prepared at 1 mg p-nitrophenol phosphate per ml in 0.1 M acetate buffer pH 5.0. A one ml substrate-enzyme mixture was incubated for a predetermined incubation period. The enzyme activity was stopped by adding to the incubation 2 ml of Tris base at 0.1 M concentration. The final optical density (OD) was measured at 410 mμ.

2. Alkaline Phosphatase.

The alkaline phosphatase was determined (Garen and Levinthal, 1960) by use of 1 mg of p-nitrophenol phosphate as substrate in 1 ml of 0.1 M Tris pH 8.5. The incubation mixture, made in 1 cm tubes, contained the following substances: 0.8 ml of substrate-buffer mixture, 0.2 ml of enzyme mixture and water. This mixture was incubated for a predetermined period of time. The reaction was stopped by adding 2 ml of Tris base to each tube and the optical density was measured at 410 mμ in a spectrophotometer. The molar absorbancy index is the same as for the acid phosphatase.

3. Other Enzymes Tested.

Blastomyces dermatitidis was cultured on CYPG medium at 37 C, the cells filtered out of the liquid, and the filtrate was tested for a number of enzymatic activities. These

were glucose-6-phosphate dehydrogenase, phosphoglucomutase (PGM), and hexokinase. All the methods used the adsorption of NADPH at 340 mμ as a measurement of the change of glucose-6-phosphate to 6-phosphogluconic acid as an assay of an enzymatic activity. The molecular change is measured at 340 mμ, where NADPH absorbs 6.2×10^3 O.D. units per mole (Worthington, 1966).

The enzyme collogenase was assayed by the technique used by Rippon and Lorincz (1964). The enzyme invertase was determined by the method of Spiro (1966). The reducing sugar was a measure of the amount of activity of the invertase.

C. Protein Determination

The protein content was determined by two methods, 1) the 260-280 adsorption ratios of Warburg and Christian (1942), and 2) the method of Lowry et al. (1951). In many samples, especially when the CYPG medium or other rich protein media were used, the protein of the solution was of no value to the experiment determined by either method.

RESULTS

Mutants of Blastomyces dermatitidis

The colony of the parent strain, B. dermatitidis St. Joseph strain, was used for comparison with the colonies that developed from the irradiated yeast cells after 10 days on CYPG medium at 37 C. Any morphological changes in the colony or changes in the rate of growth, which may indicate a nutritional deficiency, were used as a basis for selection of the isolates. A total of 45 isolates were selected from cells irradiated at 2, 4, 8, 15, 30, and 60 minutes under ultraviolet light were transferred to CYPG or BHI medium for further study. Not all of the 45 isolates grew or continued to grow after transfer. The mutants or variants were selected for isolation on the basis of change in colony morphology. Table I shows the cultures isolated with noted colony variations. Some of the 45 isolates were lost when isolated on BHI or transferred to CYPG medium which indicates unstable characteristics or deficiences in the mutants to maintain colony growth.

The remaining 26 isolates were cultured on CYPG medium at both 37 C and room temperature, 24 C. These isolates were able to grow on BHI or CYPG agar media.

Table I. The mutants of B. dermatitidis, St. Joseph strain isolated after ultraviolet irradiation on CYPG medium at 37 C.

Mutant	Exposure min.	Colony Appearance
M-3	60	crenulate edges
M-12	2	folded waxy
M-13	2	folded waxy
M-14	2	folded waxy
M-17	2	waxy, folded
M-24	4	wrinkled waxy
M-25	4	some reddish color
M-26	4	slow growing
M-27	4	wrinkled, waxy
M-28	4	crenulated edges
M-29	4	slow growing
M-30	4	small projections
M-31	4	normal
M-32	4	wrinkled, waxy
M-33	4	crenulated
M-34	4	crenulated
M-35	4	wrinkled, waxy
M-36	4	wrinkled, waxy
M-37	4	variated yeast
M-38	4	wrinkled, waxy
M-39	4	wrinkled, waxy
M-40	4	whitish-rose, granulated
M-41	4	crenulate edge
M-42	4	wrinkled edges
M-43	4	slow growing
M-44	4	small and crenulate edges
St. Joseph	0	wrinkled, waxy

A. Mutant M-36

Figure 1 will demonstrate the change that has occurred with the organism isolated from its colony. The dimorphic organism B. dermatitidis has lost its ability to change to mycelium phase. This fact can be confirmed by observing the structure of a room temperature culture (supplement aBM) under the microscope (Figure 2). Note the absence of any hypha in M-36; while the parent strain has many true hypha, with chlamydospores and other factors which are typical of B. dermatitidis grown at 24 C.

After mutant M-36 was grown on CYPG medium at 24 C and 37 C for up to 30 days, the colony appearance at both temperatures remained in the yeast-like form with a wrinkled, waxy appearance. The usual downy to granular appearance of the mycelial phase did not appear except for an appearance in a sector. Figure 1 illustrates the colony of the parent B. dermatitidis St. Joseph strain, and the colony of the mutant, M-36.

When the parent strain was compared microscopically with the mutant M-36 (Figure 2), the parent had the usual hyphae, conidia and chlamydospores while the mutant had typical thick-walled budding cells, 8-15 μ in diameter. Some of the cells, not more than 3-4, would remain together.

Figure 1. Mycelial phase colony of B. dermatitidis St. Joseph strain (Control) and a yeast-phase colony of mutant M-36 on CYPG medium for 7 days at 24 C.

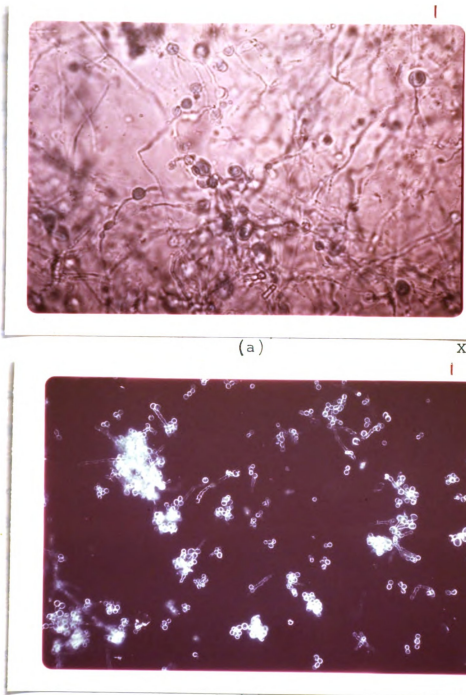

Figure 2. (a) Hyphae, conidia and chlamydospores from colony of B. dermatitidis and ; (b) budding cells of mutant M-36 on aBM medium for 20 days at 24 C.

Media for Growth of Strains of *Blastomyces dermatitidis* and Mutants

A. Amino Acids and Vitamins in Relation to Growth

The nutrient requirements of the St. Joseph strain of *B. dermatitidis* and the two morphological mutants from this strain, M-26 and M-36, were studied at 24 C. When the organisms were grown in Gilardi and Laffer's (1962) basal salts medium containing glucose and asparagine, the rate of growth of M-36 was greater than either of the other strains (see Table II). This was even more marked for M-36 with the addition of biotin and thiamine to the medium. This M-36 strain is in the yeast phase at 24 C which may relate to the more rapid growth rate. When the amino acids, tyrosine and glycine were added at 300 mg/100 ml to the basal salts medium, there was a very marked increase in the growth rate although less for M-26.

The addition of biotin and thiamine with the two amino acids to the basal salts medium had no marked effect on an increase in the growth of the strains. Halliday (1955) reported that the vitamin biotin was required for the growth; however biotin may be needed in the case of M-36, especially if other nutrients are limiting factors. Mutant strain M-26 showed less growth under these conditions

Since the rate of mycelial growth was considerably less for the M-26 strain than either of the two other strains on the basal salts medium plus the two amino acids and the two

Table II. Growth of B. dermatitidis St. Joseph strain and mutants M-26 and M-36 on Gilardi and Laffer's medium with asparagine (aBM), certain amino acids, and vitamins for 20 days at 24 C.

Strain	Medium	pH	Wet Wt. mg
St. Joseph	Basal	6.3	104.5
M-36	Basal	7.3	220.0
M-26	Basal	6.7	108.7
St. Joseph	Biotin + Thiamine[1]	6.2	93.0
M-36	Biotin + Thiamine[1]	7.4	322.0
M-26	Biotin + Thiamine[1]	6.6	114.7
St. Joseph	Glycine + Tyrosine[2]	7.4	1496.0
M-36	Glycine + Tyrosine[2]	7.8	1585.3
M-26	Glycine + Tyrosine[2]	7.0	1070.0
St. Joseph	2 Amino Acids + 2 Vitamins[3]	7.6	1596.7
M-36	2 Amino Acids + 2 Vitamins[3]	7.8	1426.7
M-26	2 Amino Acids + 2 Vitamins[3]	7.0	737.3

[1]One microgram of biotin + 10 μg thiamine/100 ml incubation.

[2]Three hundred mg of the amino acids tyrosine and glycine per 100 ml.

[3]Add 300 mg of tyrosine and glycine plus 1 μg of biotin and 10 μg of thiamine per 100 ml.

vitamins, it was thought that perhaps one of the other amino acids was required for the mycelial growth. In view of this, a series of 24 amino acids (100 mg) were added to each 100 ml of medium in 24 of these flasks for comparison with controls. The results of these experiments showed that in all cases there was no growth of the organism at 24 C. This indicated that strain M-26 requires more than a single amino acid and there are other limiting factors for growth.

Additional media were compared with that of Gilardi and Laffer (1962) for rate of growth of the St. Joseph strain and a strain from Georgia, Ga-1. These media included those of Salvin (1949), Levine and Ordal (1946), and CYPG medium of Beneke, Wilson and Rogers (1969). The results are shown in Table III for St. Joseph strain and Table IV for Ga-1 strain.

The data in Tables III and IV indicate that CYPG is the most complete enriched culture medium for the organism at 24 C. The Levin and Ordal (1946) medium is a more nearly complete medium for the growth requirements of the organism at 37 C. The BM plus asparagine also shows a good growth at 37 C. However, the CYPG medium will support very good growth of the organisms in stock cultures at both temperatures.

B. Proline and Asparagine in Relation to Growth and Viability

When proline was added with or without asparagine in the basal salt medium of Gilardi and Laffer, a marked variation occurred for the St. Joseph strain. The cell viability

Table III. Growth of B. dermatitidis St. Joseph strain on several basic media for 15 days at 24 and 37 C.

Medium	Temp.	Dry Wt.[1] mg	pH
Gilardi and Laffer[2]	24	13.0	5.8
CYPG	24	815.5	8.1
Salvin	24	113.5	5.6
Salvin	37	126.0	5.3
Levine and Ordal	37	333.0	5.3

[1]Usually based on 4 replicates.

[2]Basal medium.

Table IV. Growth of B. dermatitidis Ga-1 strain on several basic media for 15 days at 24 and 37 C.

Medium	Temp.	Dry Wt.[1] mg	pH
Gilardi and Laffer	24	35.0	5.4
CYPG	24	347.3	8.3
Gilardi and Laffer (BM + asparagine)	24	58.0	6.0
Gilardi and Laffer (BM + asparagine)	37	165.5	7.3
Levine and Ordal	37	270.0	6.0

[1]Usually based on 4 replicates.

was lost rapidly in the basal medium alone at 37 C. A great increase in the yeast phase (37 C) growth occurred in the basal medium with asparagine and proline, while without asparagine this did not occur (see Table V). However, there was no marked change in growth of the mycelial phase with proline and asparagine for the Ga-1 strain at 24 C. This also is true of St. Joseph strain at 24 C.

Table V. Growth of St. Joseph strain and Ga-1 strain of B. dermatitidis on modifications of Gilardi and Laffer's medium.

Strain	Medium	Temp.	pH	Dry Wt.[1] mg
St. Joseph	BM[2]	24	4.9	32
St. Joseph	BM	37	3.3	50
St. Joseph	BM + Proline	24	6.3	113.2
St. Joseph	BM + Proline	37	3.8	89
St. Joseph	aBM + Proline	24	7.2	120
St. Joseph	aBM + Proline	37	5.2	285
Ga-1	BM	24	3.9	44.6
Ga-1	aBM + Proline	24	6.1	52.0

[1]Usually based on 4 replicates.

[2]BM = Basic medium of Gilardi and Laffer (1962).
aBM = Asparagine + basic medium.

When using Galardi and Laffer BM and BM medium with asparagine to determine growth rate of the St. Joseph strain at 37 C during a 24-day period, the viability of the cells

was checked using the Janus green method. Figure 3 shows the growth rate on basal salts medium (BM) and on the BM plus asparagine medium (aBM). There was a dramatic change in viability after the seventh day in the BM cultures, and by the eleventh day most of the cells were dead. The viability of the cells in the aBM medium remained near 90% at the end of the 24 day period.

Table VI demonstrates that in the BM medium at 37 C with the addition of proline there is an increase in cell weight of the organism, but without asparagine in the medium the dry weight and the number of viable cells are greatly reduced. Figure 4 shows a relation of time to the number of viable cells and growth as measured by optical density at 550 mμ. In this data a greater growth occurred in the CYPG medium, while there was greatly reduced cell growth in other media.

The growth curve of B. dermatitidis was established (see Figure 5) for the St. Joseph strain in a CYPG medium cultured at 24 and 37 C. The cell wet-weight at 24 C reached a growth peak of about 3.5 g per 100 ml of medium in 14 days. At 37 C the yeast wet-weight reached a peak of 2.5 g per 100 ml of medium in 10 days.

Different amino acids were added singly and in combination to the basic salts medium of Gilardi and Laffer (1962), to determine the cell growth and viability of yeast cells of B. dermatitidis St. Joseph strain at 37 C. The growth and viability of the yeast cells remained high when asparagine was present in the medium except in one case. In this

Figure 3. The growth rates were measured by O.D. and viability of cells was determined for B. dermatitidis St. Joseph strain on basal salts medium (BM) and BM plus asparagine (aBM) at 37 C.

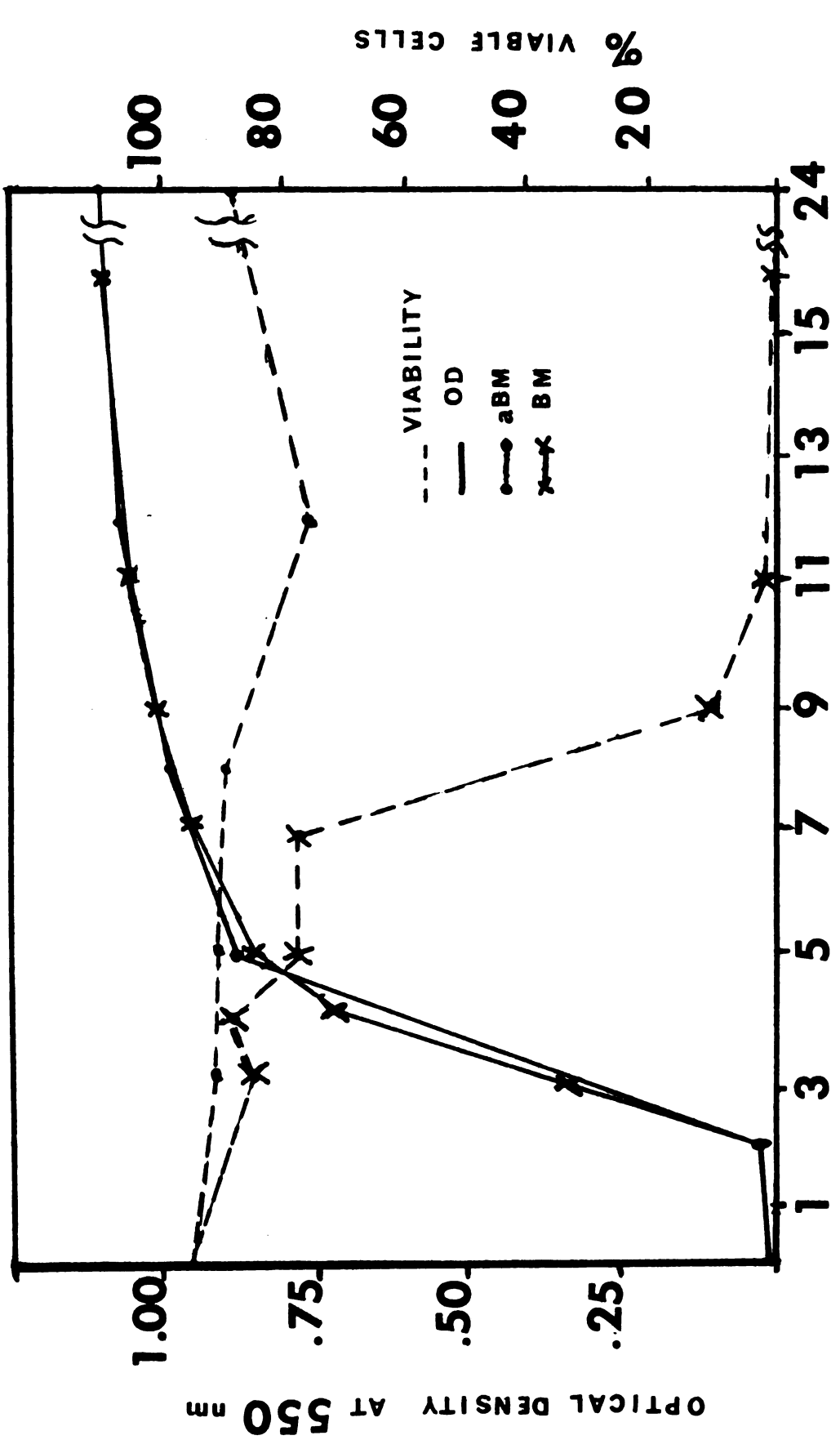
OPTICAL DENSITY AT 550 nm
1.00
.75
.50
.25
% VIABLE CELLS
100
80
60
40
20
1
3
5
7
9
11
13
15
24
DAYS CULTURED AT 37°C
VIABILITY
OD
aBM
BM

FIGURE 3

Table VI. Growth rates and viability of B. dermatididis St. Joseph strain on different media after 15 days incubations at 37 C.

Medium	Dry Wt. mg	pH
CYPG	451	8.7
aBM + Proline	285	5.15
BM + Proline	89	3.6
BM	43	3.5

Table VII. Cell growth and viability of yeast cells of B. dermatitidis St. Joseph strain on the basic salts medium of Gilardi and Laffer with different amino acids. Cultured at 37 C.

	Day 5			Day 8			Day 12		
Amino Acid	OD[1]	%V[2]	DW[3]	OD	%V	DW	OD	%V	DW
None	0.39	71	--	0.60	11	42	0.60	1	--
Asparagine	0.88	91	--	1.00	90	179	1.08	77	160
Proline	0.77	95	--	0.58	49	56	0.70	31	90
Asparagine + proline	0.46	78	--	0.95	75	166	1.10	82	180
Proline + Glycine	0.23	86	--	0.50	81	65	0.66	4	--
Asparagine + Proline + Glycine	0.92	93	--	0.92	75	298	1.13	97	224
Glycine + Tyrosine	0.15	94	--	0.33	78	55	0.36	91	53
Asparagine + Glycine + Tyrosine	0.28	88	--	0.47	87	86	0.51	71	73

[1]OD = Optical density at 550 mμ.

[2]%V = Percent viability.

[3]DW = Dry weight.

Figure 4. The growth rates were measured by O.D. at 550 mμ and the viability of cells was determined for B. dermatitidis, St. Joseph strain with proline (PRO) in basal salts medium (BM) and BM plus asparagine (aBM) in comparison with CYPG medium.

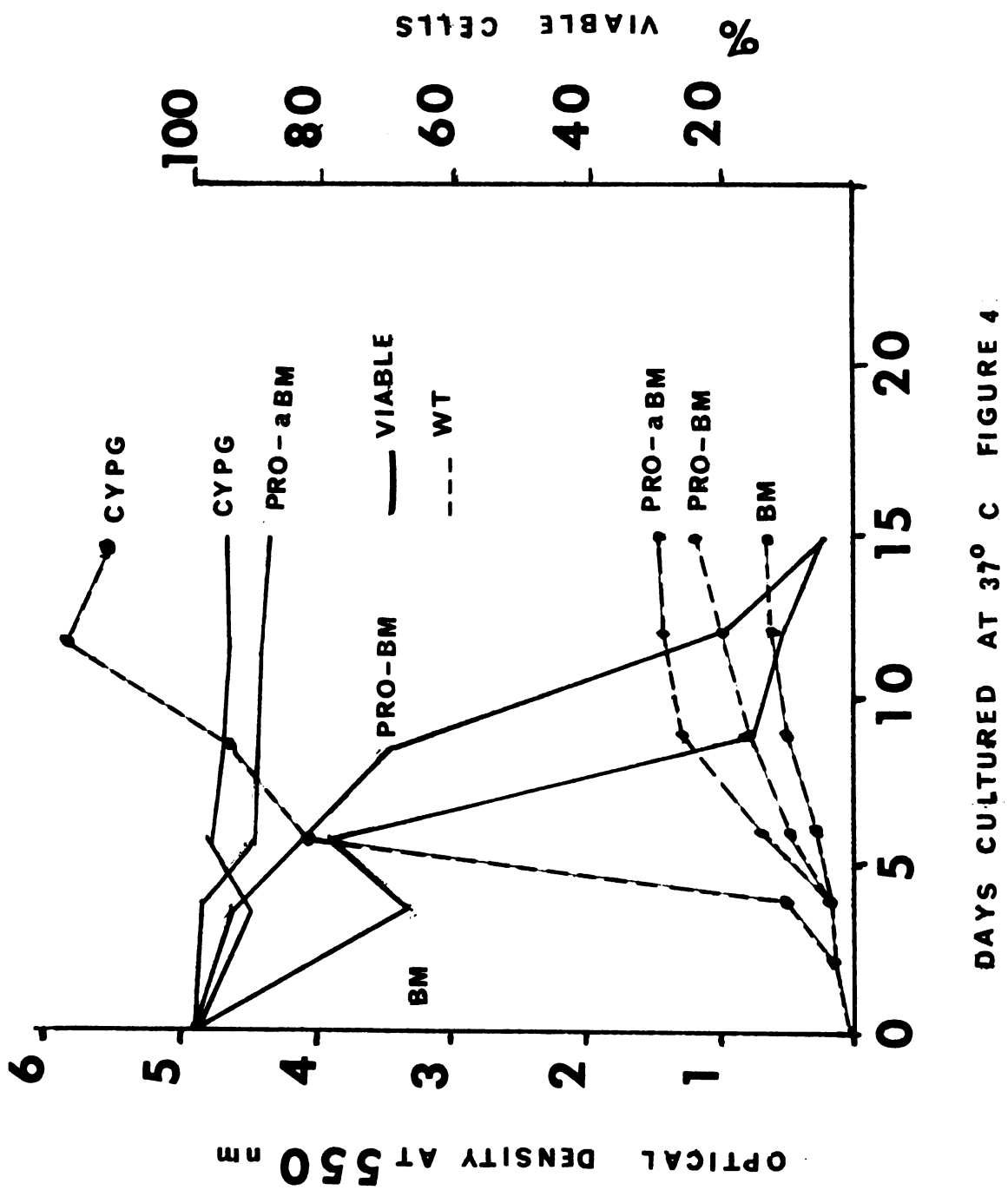
OPTICAL DENSITY AT 550 nm
VIABLE CELLS %
DAYS CULTURED AT 37° C
CYPG
CYPG
PRO-aBM
VIABLE
WT
PRO-BM
BM
PRO-aBM
PRO-BM
BM

FIGURE 4

Figure 5. Growth curves of B. dermatitidis in the mycelium (24 C) and the yeast (37 C) phase on a CYPG medium.

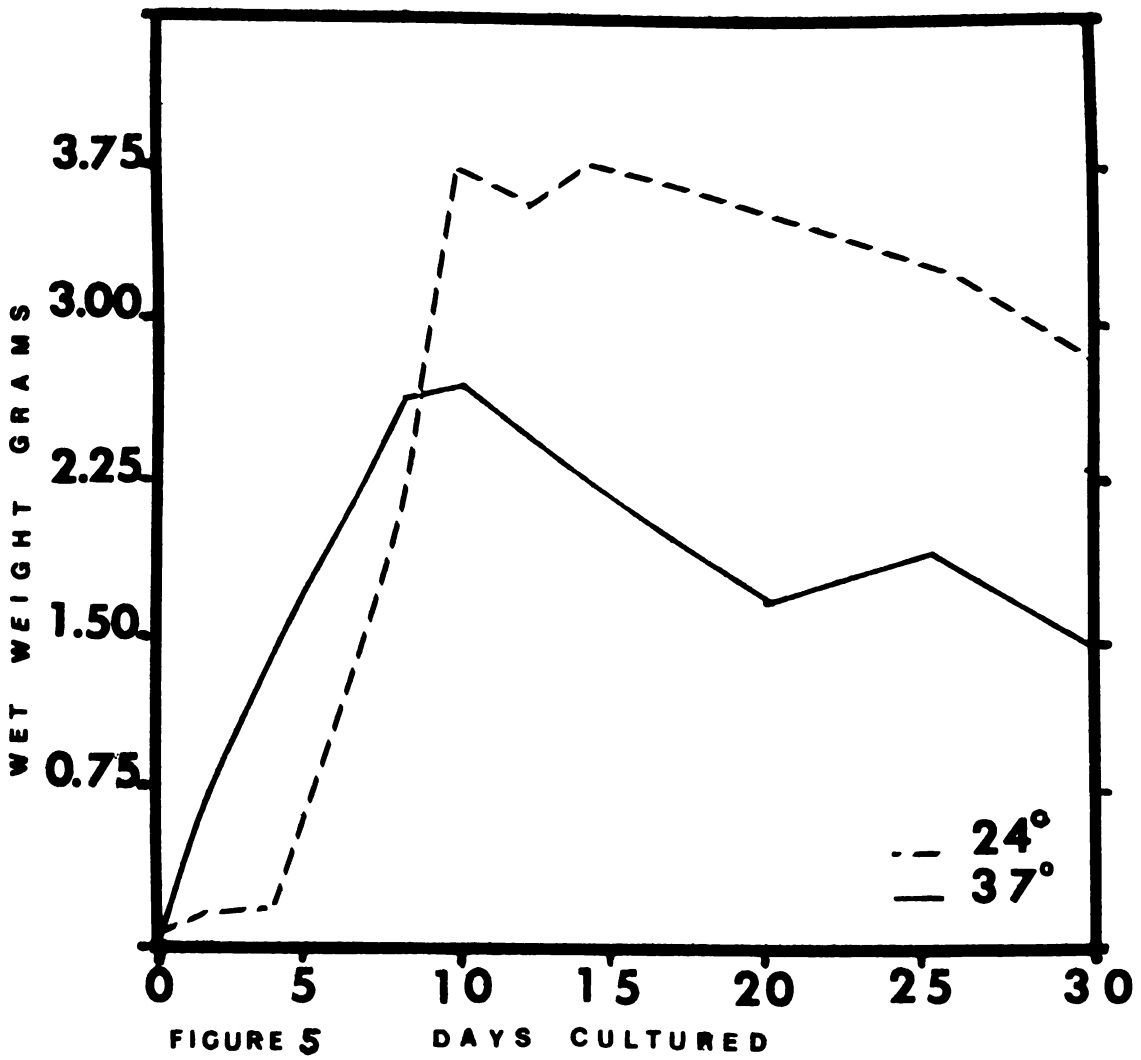
WET WEIGHT GRAMS
3.75
3.00
2.25
1.50
0.75
0
5
10
15
20
25
30
DAYS CULTURED
24°
37°

FIGURE 5

situation, no cell increase occurred with the asparagine, glycine and tyrosine combination, while good growth occurred when proline was substituted for glycine (see Table VII). This organism was not able to utilize glycine in combination with tyrosine in the medium at 37 C while at 24 C excellent mycelial growth developed (see Table II) with glycine, tyrosine along with biotin and thiamine. This indicates a marked difference in utilization of the two amino acids at 24 and 37 C for this diphasic organism.

Virulence of the Mutant Strains

The virulence of the mutant strains of B. dermatitidis St. Joseph was determined by intraperitoneal inoculation of the yeast phase of the organisms. The days to 50% kill were determined when possible for each mutant strain, at a higher concentration of cells of about 5×10^6. The usual LD_{50} for the parent St. Joseph strain would be around 14 to 16 days. At a dilution of 5×10^5 thirty days was necessary for an LD_{50}. Table VIII shows the preliminary test for virulence of 21 of these mutants. These mutant organisms showed a range of virulence; for instance, M-24 and M-42 and M-43 were nearly or slightly more virulent than the parents. A less virulent group of organisms included M-3, M-35, and M-36, with the latter two appearing to be less virulent as higher cell concentrations were used. The third group of organisms appeared less virulent, as M-26 produced no symptoms in the mice at a cell dilution of 4×10^5.

Table VIII. Virulence of mutants of the St. Joseph strain of *B. dermatitidis* injected intraperitoneally (IP) into mice.

Mutant	Inoculum	Days to 50% Kill	No. Mice Killed	Observations
St. Joseph	5×10^5	30	5/5	granulomas
M-3	7.5×10^5	45	4/5	granulomas
M-12	6×10^5	36	4/5	granulomas
M-13	5.2×10^6	23	5/5	granulomas
M-14	9.85×10^6	22	5/5	granulomas
M-17	2.4×10^6	23	6/6	granulomas
M-24	6.5×10^5	24	5/5	granulomas
M-26	4×10^5	--	0/6	no granulomas
M-27	1.1×10^7	12	5/5	granulomas
M-28	5.2×10^6	16	6/6	granulomas
M-29	5.2×10^7	14	4/5	granulomas
M-30	1.0×10^6	26	5/5	granulomas
M-31	1.04×10^7	17	5/5	granulomas
M-32	6.4×10^6	14	5/5	granulomas
M-35	1.0×10^6	46	3/5	granulomas
M-36	4.0×10^6	45	6/8	granulomas
M-38	5.65×10^6	26	5/5	granulomas
M-40	8.0×10^4	--	1/5	granulomas
M-42	1.0×10^5	34	4/5	granulomas
M-43	5.0×10^5	39	4/5	granulomas
M-25	4.25×10^6	--	2/5	granulomas
M-37	5.4×10^6	--	0/5	no granulomas

Two other mutants, M-25 and M-37, were less virulent at a dilution of 4.25×10^6 and 5.4×10^6 respectively. Higher concentrations need to be used to determine the days to 50% kill.

Other strains of B. dermatitidis were selected for virulence tests. The 12 strains from patients in different parts of the United States show a variation in virulence when injected into mice (see Table IX). For example, the Ga-1 strain from Georgia had no days to 50% kill at 1.4×10^5 and was selected for further tests at higher cell concentration (see Table XI). The Oklahoma, Kl-1, 130, and S-182 strains are examples of less virulent strains of B. dermatitidis in comparison to St. Joseph strain or several other more virulent strains tested.

A. Virulence of the Mutant M-26

A comparison was made on the effect of increasing cell concentration of the mutant M-26 and the parent St. Joseph strain on virulence in mice. Table X shows the difference in virulence of M-26 in comparison to the parent strain of St. Joseph. The parent strain had 29 days to 50% kill with 1.1×10^5 cells of inoculation in the mice, while the M-26 mutant required 30 days with a 1.2×10^7 cells of inoculation in the mice. For a 50% kill in 30 days in the mutant strain it is necessary to have over 100 times as much inoculum as is required in the parent strain. This makes the mutant an interesting organism for study of factors related to virulence. Table XI shows a

Table IX. The relative virulence of twelve strains of Blastomyces dermatitidis, injected IP in mice.

Strain	Inoculuation	No. Mice Killed	Days for 50% Kill
St. Joseph	9.4×10^5	6/6	22
Wisconsin-788	1.0×10^6	5/6	24
Wisconsin-784	3.2×10^5	4/6	29
Ga-1	1.4×10^5	0/7	--
SL-1	1.2×10^5	4/6	26
KL-1	2.6×10^5	1/6	--
130	7.5×10^5	1/6	--
S-182	1.5×10^6	0/6	--
Oklahoma	3.75×10^7	2/4	89
Van Camp	3.28×10^7	4/4	25
Duke	2.1×10^7	7/8	45

Table X. The effect of increasing cell concentrations of the mutant M-26 of B. dermatitidis on virulence when injected IP in mice.

Strain	Viable Inoculum	Wet wt. mg	Days for 50% Kill	No. Mice Killed	Observations*
Parent	1.55×10^7	14.1	14	4/4	Granulomatous
Parent	1.1×10^6	1.0	21	4/4	Granulomatous
Parent	1.1×10^5	0.1	29	4/4	Granulomatous
M-26	1.2×10^7	20.8	30	4/4	Granulomatous
M-26	5.8×10^5	1.0	--	0/4	No granulomas
M-26	5.8×10^4	0.1	--	0/4	No granulomas

*The experiment was terminated after 90 days.

NOTE: M-26 at 8.2×10^6 cells per mouse had 6 mice killed out of 16.

comparison of serial dilutions of yeast cells of strains Ga-1, M-37, M-25, and St. Joseph. The mutant M-37 was less virulent than the mutant M-25 and the Georgia strain (Ga-1) as indicated by the days for 50% kill at a dilution of 10^8 cells.

Table XI. Effect of dilutions of cells of different mutants and strains of B. dermatitidis on virulence when injected IP in mice.

Organism	10^8	Days for 50% Kill	10^7	Days for 50% Kill	10^6	Days for 50% Kill	10^5	Days for 50% Kill
Ga-1	6/10*	21	0/10	--	0/10	--	--	
M-37	5/8	78	0/10	--	0/10	--	--	
M-25	6/7	13	1/10	--	2/10	--	--	
St. Joseph	--	--	4/4	14	4/4	16	9/9	30

*Number of mice killed/total number of mice.

NOTE: M-26 at 8.2 x 10^6 cells per mouse had 6 mice out of 16 killed at the end of 90 days.

B. Toxin as a Factor

A toxin determination was made with the St. Joseph strain by the method similar to that reported by Taylor (1964). He indicated that a toxin may be the cause of death of the mice in his experiments. Table XII shows the results of the toxin experiment indicating that trypsin-treated cells did give a more rapid kill of the mice than did the control. Treatment with 1% HCl solution, however, required more time for the kill than did the control. One control for trypsin that was

Table XII. A test for toxin production in B. dermatitidis St. Joseph strain grown at 37 C. Inoculation included 2 mg of acetone-dried tubercle bacilli/mouse plus the treatment.

Treatment	Inoculation mg	No. Mice	Hours to 50% Kill
37 C Control	40	7/7	156
37 C Trypsin[1]	40	7/7	72
37 C 1% HCl[2]	40	7/7	192
65 C HK[3] Trypsin	40	0/7	--
65 C HK Control	40	0/7	--
Saline	0	0/7	--

[1]Two mg of trypsin treatment for 1 hour at 37 C.

[2]One percent concentrated HCl solution treatment for 1 hour at 37 C.

[3]HK = Heat kill at 65 C for 1 hour.

not run by Taylor was added to these tests to see if the trypsin-treated heat-killed cells would cause death of mice. No mice were killed under these conditions. The high number of yeast cells in the trypsin treated mice in comparison to the control mice does not necessarily indicate toxin later on in the experiment. However, in the time period up to 96 hours after inoculation, a toxin could account for the more rapid death rate in the animals. A second experiment was run in which there was a reduction in the amount of trypsin-treated cells inoculated into the animals. In this case all the animals died at the same rate as the control animals.

An experiment similar to Salvin (1952) was tried with 2 mg of heat-killed and 2 mg of acetone-killed cells per mouse. None of these animals died, however, the control animals with 2.8×10^6 cells per ml had an LD_{50} of 17 days. This evidence suggested that there was no toxin, or not sufficient toxin in the strain used to kill the animals as was reported by Salvin (1952).

C. Lipids Effect on Virulence

A higher lipid content of strains of B. dermatitidis has been reported by DiSalvo and Denton (1963) to represent an increase in virulence. The organisms were grown in BHI plus biotin agar for 10 days at 37 C. The lipid content of four strains, St. Joseph, Oklahoma, Van Camp, and Duke was extracted with chloroform-methanol (2:1) for 48 hr, for animal inoculation. After intraperitoneal

inoculation of cells of the four strains into mice, the Oklahoma strain with the high lipid content had a 50% kill of 89 days (see Table XIII). The strain with the highest lipid content had a 50% kill of 63 days while the other two strains with lower lipid contents had more rapid kill indicating greater virulence.

Three strains were selected for further comparison of lipid concentration in the cell with virulence in the animal. Two of the strains, M-26 and Ga-1, were low in virulence compared to the St. Joseph strain which higher had higher virulence. The Al-Doory and Larsh (1962) procedure under Methods was used to determine lipids. These organisms were grown on the enriched CYPG medium for 15 days at 37 C. Table XIV shows the lipid content is higher for the two less virulent strains, M-26 and Ga-1; lower for the more virulent St. Joseph strain. Table XV shows the lipid content for two of the above strains, St. Joseph and Ga-1 strain still has a higher lipid content similar to the St. Joseph strain with a higher lipid content at 24 C than at 37 C. This result differed from that reported by Al-Doory and Larsh (1962).

Alkaline and Acid Phosphatase Activities

A. Cultures at 24 and 37 C Assayed for Enzyme Activity.

Variations occurred in the amount of acid and alkaline phosphatase activity produced by the various mutants and

Table XIII. Days to 50% kill and lipid concentration of four strains of *B. dermatitidis* after a culture on BHI plus biotin agar for 10 days at 37 C. The cells were extracted with chloroform-methanol (2:1) for 48 hr on a soxhlet.

Organisms	Wt. in mg			% Lipids	Inoculation	No. Mice Killed	Days to 50% Kill
	Wet	Dry*	Lipid*	Total*			
St. Joseph	915.9	186.5	9.84	5.87	1.64×10^7	4/4	14
Oklahoma	802.8	162.1	12.77	9.49	3.75×10^7	2/4	89
Van Camp	904.8	189.4	5.28	3.49	3.28×10^7	4/4	25
Duke	966.6	151.1	16.97	11.30	2.10×10^7	4/4	63

*Average of two determinations.

Table XIV. The lipid concentration of the more virulent St. Joseph strain and the two less virulent strains of B. dermatitidis after culture on CYPG agar for 15 days at 37 C.

Organism	Wt. in mg			% Lipids**		
	Wet	Dry	Lipid	Free	Bound	Total %*
M-26	2486	461.7	161	14.7	20.1	34.8
Ga-1	2251	331.7	37	9.3	1.9	11.2
St. Joseph	1662	389.9	30	6.7	1.0	7.7

*Based on dry weight.

**Solvent for extraction was chloroform-methanol (2:1). Extraction was for 48 hr. The cells were treated with dilute HCl solution and re-extracted.

Table XV. The lipid content of two strains of B. dermatitidis after culture on CYPG medium for 15 days at 24 C.

Organism	Wt. in mg			% Lipids**		
	Wet	Dry	Lipid	Free	Bound	Total %*
Ga-1	3089	340.3	87	22.9	2.6	25.5
St. Joseph	6965	815.5	140	7.7	9.4	17.1

*Based on dry weight.

**Solvent for extraction was chloroform-methanol (2:1). Extraction was for 48 hr. The cells were treated with dilute HCl solution and re-extracted.

strains of B. dermatitidis. In the assay method on solid CYPG medium ultilizing plugs from the medium after growth of the organisms for 15 days, the yeast phase at 37 C, in general, showed a higher acid phosphatase activity than the mycelial phase at 24 C for all the strains checked (see Table XVI). The alkaline phosphatase activity for the organisms in the mycelial and yeast phase were somewhat similar, with some strains higher at 37 C and some higher at 24 C or nearly the same for both temperaturs. The alkaline phosphatase assay was linear for 0-0.5 O.D. at 410 mμ.

Eight strains of B. dermatitidis were grown on CYPG agar at 24 and 37 C for comparison with the mutant strains, and assayed for alkaline and acid phosphatase. The results are shown in Table XVII. There was very little difference in most of the eight strains at 24 and 37 C except for K1-1 and 130. These two strains were much higher in alkaline and acid phosphatase activity at 37 C in the yeast phase.

Filtrates from eight strains of B. dermatitidis and eight mutants grown on liquid CYPG medium for 15 days at 37 C were used for enzyme assay. The alkaline phosphatase activity was higher than the acid phosphatase in the majority of the strains including all of the mutants. These results are shown in Table XVIII. These groups of organisms were not grown at 24 C for comparison.

Table XVI. The extracellular acid and alkaline phosphatase activities of mutants of *B. dermatitidis* grown on CYPG medium for 15 days at 24 and 37 C. The activity is expressed in mμmole of substrate changed/hr/plug of agar. Average of two assays.

Mutant	Mycelial-phase		Yeast-phase	
	Acid	Alkaline	Acid	Alkaline
M-3	7.6	7.6	12.6	4.6
M-12	7.2	13.5	3.7	4.1
M-13	2.9	5.0	29.3	3.3
M-14	2.2	5.0	4.6	4.1
M-17	2.9	3.9	6.5	4.6
M-24	2.6	2.2	17.2	6.7
M-25	4.1	5.2	4.3	3.7
M-26	2.6	3.5	3.9	4.8
M-27	6.7	12.6	10.7	3.7
M-28	6.7	2.5	13.5	14.8
M-29	2.2	5.7	6.3	4.6
M-30	14.6	15.9	15.9	4.6
M-31	8.5	5.7	5.4	4.3
M-32	9.3	4.4	10.0	9.4
M-33	4.1	2.5	10.7	11.4
M-34	2.5	4.1	4.9	3.9
M-35	3.9	5.0	17.4	13.9
M-36	11.6	2.5	15.0	3.5
M-38	6.5	1.8	20.4	11.4
M-39	2.5	2.2	3.7	3.9
M-43	1.3	1.5	9.3	3.9
M-44	5.7	5.6	13.1	3.9

Table XVII. The extracellular acid and alkaline phosphatase activities of 8 strains of B. dermatitidis grown on CYPG medium for 15 days at 24 and 37 C. The activity is expressed in mμmole of substrate changes/hr/plug of agar. Average of 2 assays.

Strain	Mycelium-phase		Yeast-phase	
	Acid	Alkaline	Acid	Alkaline
St. Joseph	4.1	4.3	1.3	2.4
S-182	2.2	1.3	1.5	4.1
Ga-1	5.2	3.3	3.7	13.1
Sl-1	5.7	15.5	0.7	2.8
Wisconsin 788	3.5	6.3	1.5	2.8
Wisconsin 784	6.5	10.7	1.5	1.8
Kl-1	5.6	3.5	15.7	23.0
130	3.1	3.3	23.0	30.7

Table XVIII. The extracellular acid and alkaline phosphatase activity of 6 strains of B. dermatitidis and 8 mutant strains grown on CYPG medium for 15 days at 37 C. Filtrates were used for assays. The activity is expressed in units*/100 ml of filtrate. Average of 2 assays.

Strain	Growth	Phosphatases	
	Wet wt. in g.	Alkaline	Acid
Sl-1	1.868	30.0	17.3
Kl-1	2.814	34.7	9.4
Wisconsin 788	2.066	10.6	8.0
Wisconsin 784	1.755	5.9	7.8
130	2.784	13.6	6.1
Ga-1	1.675	13.6	6.1
M-12	2.213	24.1	13.0
M-17	2.073	37.7	15.6
M-25	1.308	30.2	11.8
M-26	1.962	36.4	17.6
M-30	2.386	33.5	14.6
M-35	1.864	42.0	17.5
M-36	1.937	19.2	15.0
M-44	2.122	16.6	8.2

*unit = 1 μmole of p-nitrophenol phosphate used per hour.

Four additional strains, two mutants and two strains of B. dermatitidis were grown in liquid CYPG medium at 24 and 37 C for 20 days. The filtrates were used for alkaline and acid phosphatase activities. The results are illustrated in Table XIX. Under these condition the organisms still produced more alkaline than acid phosphatase at 20 days but at lower levels than at 15 days. However, at 24 C both the alkaline and acid phosphatase activity was higher than at 37 C in the 20-day cultures.

Four mutants and the parent St. Joseph strain were checked for alkaline phosphatase activity and virulence in mice after the organisms were grown in CYPG medium for 15 days at 37 C. There was no definite relationship between the amount of alkaline phosphatase activity in these strains and virulence. Two strains, M-26 and M-36, with a somewhat higher enzyme activity, were less virulent (see Table XX).

B. Growth Curve of B. dermatitidis on CYPG medium at 24 and 37 C in relation to intracellular alkaline phosphatase activities.

Blastomyces dermatitidis St. Joseph strain was grown on CYPG medium at 24 and 37 C for a period of 30 days. The peak of growth for the mycelial phase was between 10 to 12 days, while the alkaline phosphatase activity from cell extracts reached a peak in 20 days (see Figure 6). The peak in growth for the yeast cells was between 9 to 10 days, which was similar time for the highest enzyme activity from cell extracts of yeast cells.

Table XIX. The extracellular alkaline and acid phosphatase activity of two strains and two mutants of B. dermatitidis, grown on liquid CYPG medium for 20 days at 24 and 37 C. Filtrates were used for assays. The activity is expressed as μmoles of substrate used/hr/100 ml. (Average of 2 enzyme assays.)

Strains	Cells, Wet wt. in mg		Phosphatases			
			Alkaline		Acid	
	24 C	37 C	24 C	37 C	24 C	37 C
Ga-1	2.84	2.525[1]	98	3	51	3
St. Joseph	3.226	1.742	20	10	15	2
M-26	4.082	2.664	23	14	17	11
M-36	1.434[1]	2.352	26	17	9	8

[1]Lost one of 2 replicates by contamination.

Table XX. The extracellular alkaline phosphatase in CYPG medium 15 days at 37 C and virulence in mice of four mutants and the St. Joseph strain of B. dermatitidis. The activity is expressed as μmole of substrate used/hr/100 ml.

Strain	Rep.	Alkaline	Wet wt. grams	No. Mice Killed	Inoculum	Days to 50% Kill
St. Joseph	5	3.6	2.21	5/6[2]	4×10^5	19
M-17	5	11.1	1.94	5/6[1]	2.4×10^6	24
M-26	5	13.0	2.84	0/6[2]	4×10^5	--
M-28	5	4.4	2.45	6/6	5.2×10^6	16
M-36	2	19.2	1.94	6/8[2]	4×10^6	45

[1]One animal died of unknown causes.

[2]Remaining animals were sacrificed after 90 days of treatment.

Figure 6. Growth curve of B. dermatitidis on CYPG medium at 24 C and 37 C for 30 days. The intracellular phosphatase activity for the yeast phase at 37 and the mycelial phase at 24 C is expressed as μmoles of substrate changed/hr/100 ml.

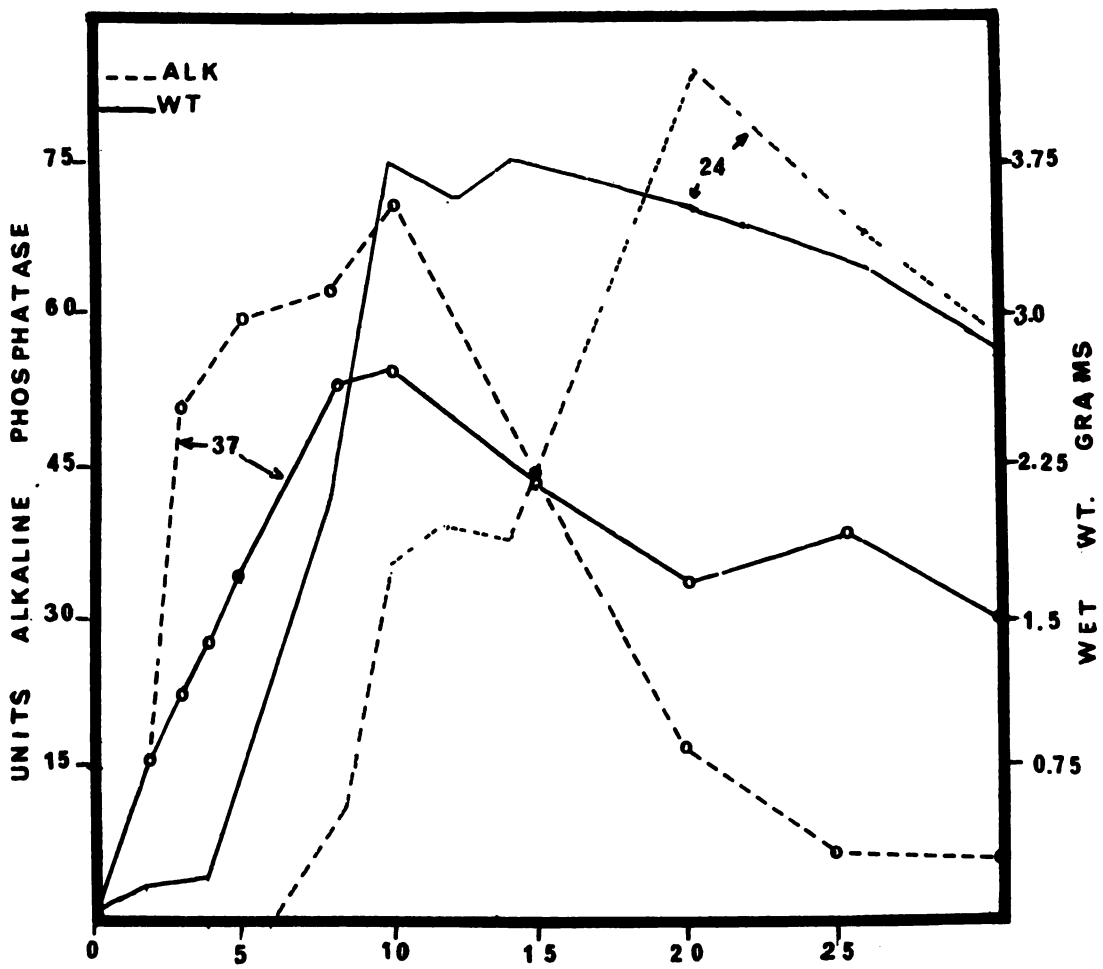
---ALK
WT
75
60
45
30
15
UNITS ALKALINE PHOSPHATASE
37
24
3.75
3.0
2.25
1.5
0.75
WET WT. GRAMS
0
5
10
15
20
25
DAYS CULTURED

FIGURE 6

C. Growth Curve of B. dermatitidis on CYPG Medium at 24 and 37 C in Relation to Intracellular and Extracellular Acid and Alkaline Phosphatase Activities.

The same St. Joseph strain was grown on CYPG medium at 37 C for a period of 30 days. The peak for the yeast phase growth, the intracellular alkaline and acid phosphatase activity in the medium was in 10 days (see Figure 7). The extracellular alkaline and acid phosphatase in the cells remained low throughout the 30-day period. The same organism after growth on CYPG medium at 24 C was checked for extracellular and intracellular acid and alkaline phosphatase activities (see Figure 8). The extracellular alkaline and acid phosphatase activities were much higher in the mycelial phase at 24 C than in the yeast phase at 37 C.

D. Phosphatase Enzymes are not Easily Released from the Cell Walls.

The acid and alkaline phosphatase enzymes of the culture media, as well as the cell wall and cell extract, were determined (see Table XXI). The culture medium contained 7.3 units of alkaline phosphatase and 5.4 units of acid phosphatase per 100 ml as expressed as μmoles of substrate changed per 100 ml incubation. After three washes were combined an additional 0.25 unit of alkaline and 0.25 unit of acid phosphatase were removed from the cell walls. The evidence in Table XXI indicates that KCl does not cause the release of any more phosphatase enzymes than does the

Figure 7. Growth curve of B. dermatitidis yeast phase on CYPG medium at 37 C, illustrating the extracellular and intracellular acid and alkaline phosphatase activities during the 30 day period. The activity is expressed as μmoles of substrate changed/hr/100 ml.

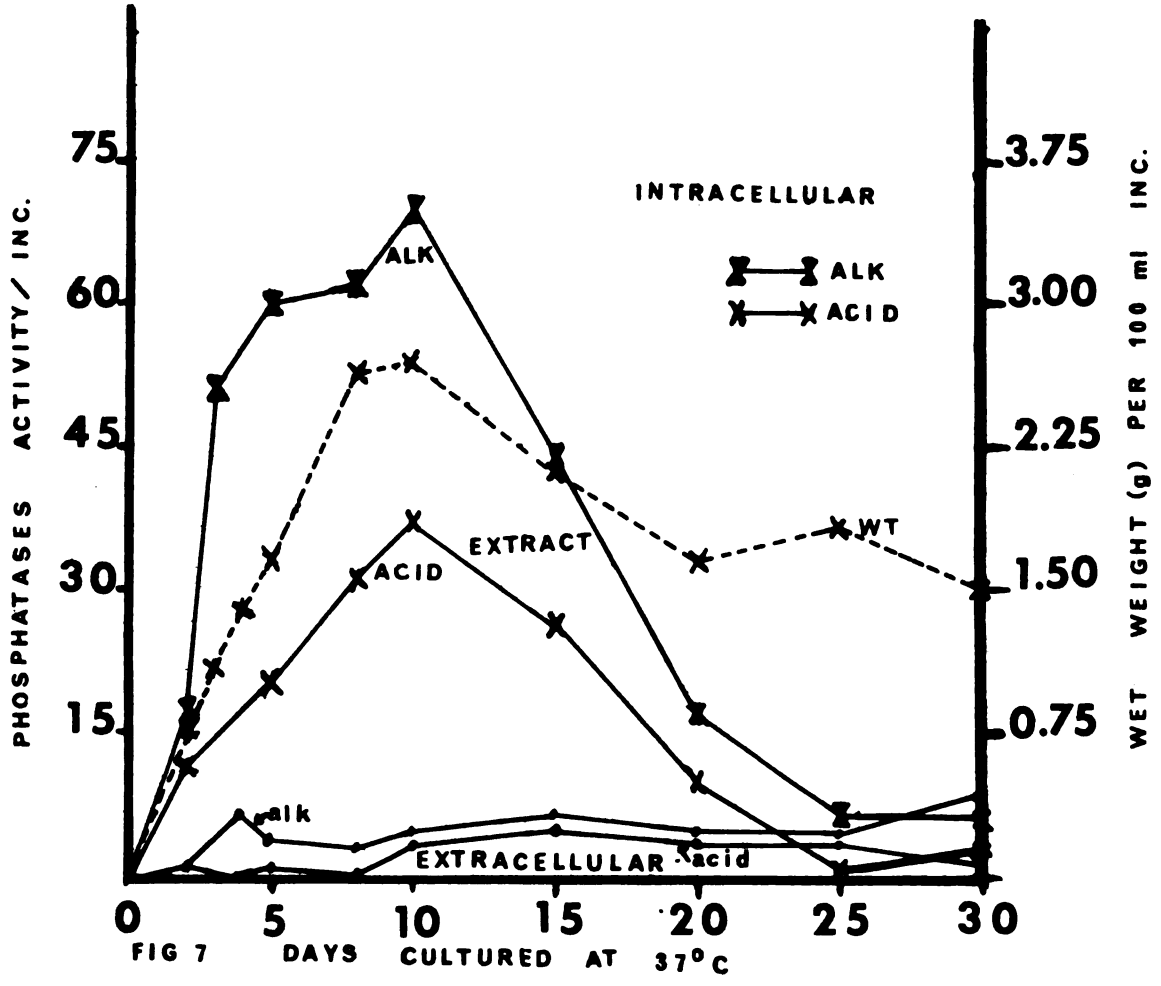
PHOSPHATASES ACTIVITY/ INC.
75
60
45
30
15
INTRACELLULAR
ALK
ACID
ALK
EXTRACT
ACID
WT
alk
EXTRACELLULAR
acid
3.75
3.00
2.25
1.50
0.75
WET WEIGHT (g) PER 100 ml INC.
0
5
10
15
20
25
30
DAYS CULTURED AT 37°C

FIG 7

Figure 8. Growth curve of B. dermatitidis mycelium on CYPG medium at 24 C illustrating extracellular and intracellular acid and alkaline phosphatase activities during the 30 day period. The activity is expressed as μmoles of substrate changed/hr/100 ml.

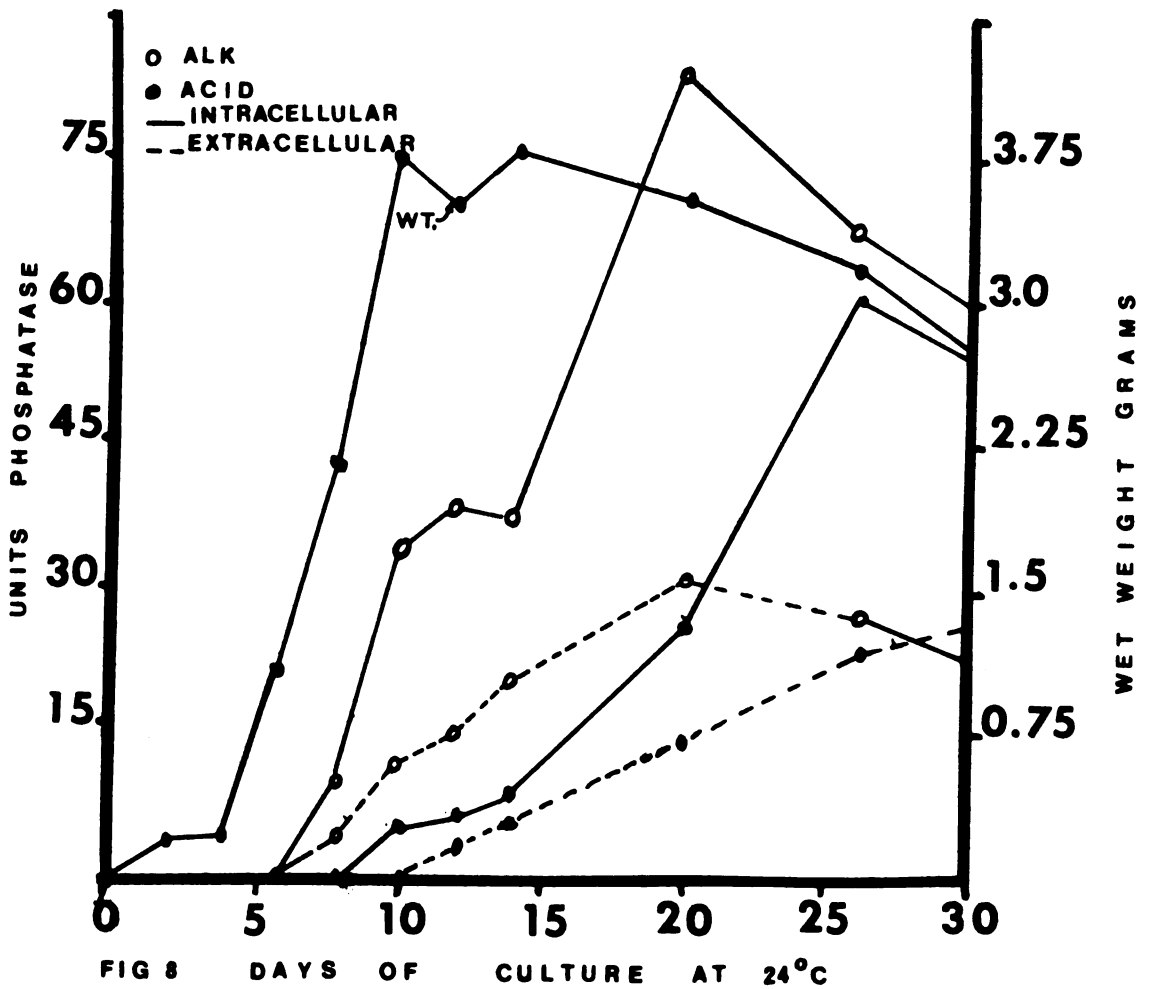
O ALK
● ACID
—INTRACELLULAR
– –EXTRACELLULAR
WT.
UNITS PHOSPHATASE
75
60
45
30
15
3.75
3.0
2.25
1.5
0.75
WET WEIGHT GRAMS
0
5
10
15
20
25
30
DAYS OF CULTURE AT 24°C

FIG 8

Table XXI. The release of acid and alkaline phosphatase enzymes from cell walls of B. dermatitidis in high salt concentration vs distilled water controls. The units of enzyme activity are expressed as μmoles/hr at 30 C.

Treatment	KCl		Water	
Wet Cells in grams	4.506		1.294	
	Alkaline	Acid	Alkaline	Acid
Extracellular enzymes, after 4 hrs in KCl or H_2O	2.0	2.6	0	0.3
Total Cell Extract Activity	68.0	15.7	14.1	3.9
Activity/gram of cells	15.7	3.5	10.9	3.0
Ultracentrifuged, activity/gram of cells	14.2	3.5	7.4	2.5

washing of cells with water. The release of proteins was greater in relation to cell weight in the case of KCl extraction (Weimberg, 1966), but the acid or alkaline phosphatase activity was about the same. Some authors have suggested that phosphatases are associated with cell membranes. In this experiment, after the cell extract was ultracentrifuged at 105,000 X g for 2 hours to see if the enzymes were particle bound, there was only a small loss of enzyme activity in the supernatant (see Table XXI).

E. Location of Alkaline Phosphatase Activities in Polyacrylamide Gels.

A modification of the Davis method (1951) was used. After the proteins from the 20-day-old cell-free CYPG culture medium at 24 and 37 C were lyophilized, a 15 mg sample of the dried material was used per gel. Figure 9 shows a diagram of the Commassie blue stained electrophoretic protein patterns of St. Joseph strain and a less virulent mutant, M-26. The cut gels, 1 cm apart, unstained, show That at 20 days the alkaline phosphatase activity of the 37 C culture filtrates, when checked spectrophotometrically, are much lower than the culture filtrates at 24 C. At 24 C a higher alkaline phosphatase activity is noted especially between the 2 to 3 cm range of the gel. This data supports the results shown in Figures 7 and 8, in respect to the alkaline phosphatase enzymatic acitivity under similar sulture conditions.

Figure 9. Diagrams of electrophoretic patterns in polyacrylamide gels of lyophilized filtrates of mutant M-26 and St. Joseph strain of B. dermatitidis. Alkaline phosphatase activity measured spectrophotometrically at 410 mμ. Cultures grown on CYPG medium for 20 days at 24 and 37 C.

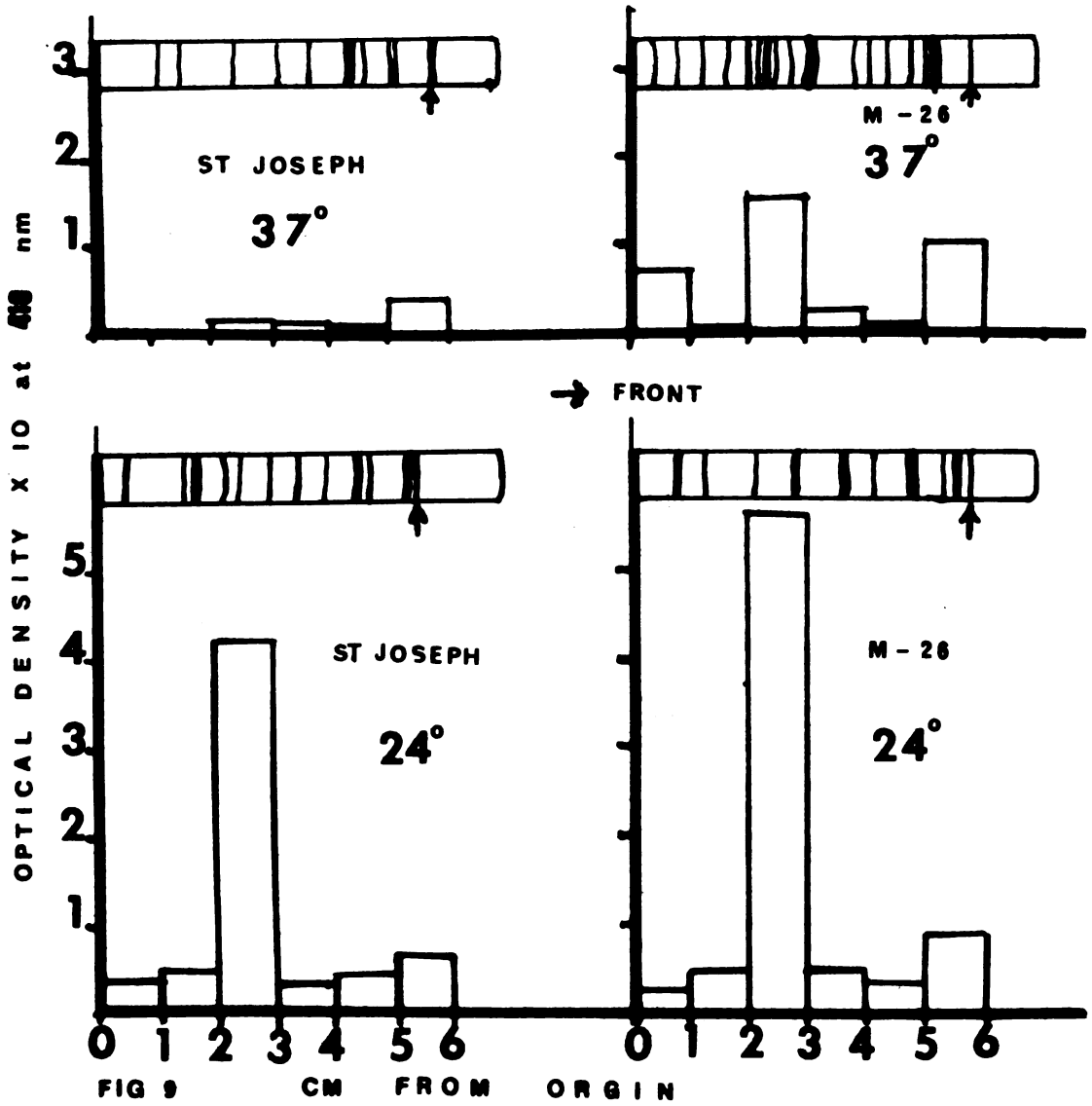
ST JOSEPH
37°
M - 26
37°
FRONT
ST JOSEPH
24°
M - 26
24°
OPTICAL DENSITY X 10 at nm
0 1 2 3 4 5 6
0 1 2 3 4 5 6
CM FROM ORGIN

FIG 9

Other Enzyme Activities of Blastomyces dermatitidis, St. Joseph Strain

The 10-15 day liquid culture filtrate of B. dermatitidis was examined for a number of other enzymatic activities. The following assays were used to determine glucose-6-phosphate dehydrogenase, phosphoglucomutase and hexokinase. Excessive amounts of phosphoglucomutase and glucose-6-phosphate dehydrogenase were used to determine hexokinase; only glucose-6-phosphate dehydrogenase was added to the reaction mixture to determine phosphoglucomutase; and no additions were required to determine glucose-6-phosphate dehydrogenase. The substrates for hexokinase were glucose, adenosine triphosphate (ATP) and NADP; for phosphoglucomutase, glucose-1-phosphate and NADP; and for glucose-6-phosphate dehydrogenase, glucose-6-phosphate and NADP. The assay used NADPH formed from the reaction:

glucose-6-phosphate + NADP $\longrightarrow$ 6-phosphogluconic acid + NADPH

This product (NADPH) was measured at 340 mμ in a Beckman Model DU spectrophotometer equipped with a Gilford automatic sample changer and recorder (Wood and Gilford, 1961). No activity of these enzymes could be seen in the 10-15 day culture filtrate, even though the cell extract showed enzymatic activity.

The culture filtrate was tested for collogenase and invertase. No collogenase was found. Spiro's method (1966), based on

reducing sugars, was used. The culture filtrate, which contained high levels of reducing sugars, makes this method of no value.

The alkaline and acid phosphatases were the only enzymes with detectable levels of activity in the supernatant liquid to examine further for possible correlation to virulence.

DISCUSSION

Strains of Blastomyces dermatitidis vary for virulence in laboratory animals. Some strains are more virulent in mice than others. Since avirulent strains are desirable for the study and development of viable vaccines, the development of avirulent mutants would be desirable.

A number of stable strains of mutants were produced by ultraviolet light, from the St. Joseph strain of B. dermatitidis. The mutants were changed morphologically as evidenced by modifications in colony appearance, rate of growth, and color changes, or by changes in physiological requirements. One extreme change in the normally diphasic condition of the fungus was the yeast-phase maintenance colony at both 24 and 37 C in mutant M-36. Some mutants that received sublethal doses of ultraviolet light were able to grow for only a short period of time after the first transfer in the culture media. The mutants selected for enzyme, nutritional and virulence studies remained stable.

In a survey of the literature, there appear to be no previous references on development of mutants in B. dermatitidis. Hollaender and Emmons (1946) were among the early investigators to report that some of the ringworm pathogens, as

Trichophyton mentagraphytes, produced mutants closely resembling naturally occurring species. While in other cases modifications were sufficient to be classified as new "species" if they had not augmated from specific fungus cultures. Recently Walch (1970) reported on the use of induced mutants of Coccidioides immitis for immunization studies in mice. The most modified change in B. dermatitidis was to a yeast-type colony in this investigation, at both temperatures. This has some resemblence to other genera and species of yeasts but virulence and morphological appearance at 37 C in this case are sufficient to establish the mutant as a variation of B. dermatitidis.

Nutritional investigations of the mutants were made for comparison with strains of B. dermatitidis. Mutant M-36 was of special interest as the yeast phase at 37 C continued to grow in culture at 24 C while the normal strains are diphasic, becoming mycelial at 24 C. Temperature is considered the main factor in the transformation of the mycelial phase to the yeast phase at 37 C (Nickerson and Edwards, 1949). In the case of the mutant M-36, the failure to convert to the mycelial phase may be due to a modification of an enzyme.

Although a number of media may be used for growth of the mycelial and yeast phases, the selection of a defined medium suitable for nutrient studies of both phases of the mutants was desired. The mycelial form has been grown on media containing organic nitrogen sources such as peptone

casein hydrolysate (Levine and Ordal, 1946), sulfate asparagine (Area and Cury, 1950), and glycine (Nickerson and Edwards, 1949). However, the mycelial form can utilize ammonium salts in place of organic nitrogen. The yeast phase had been reported by Gilardi and Laffer (1962) to assimilate 21 carbon and 25 nitrogen substrates in liquid or shake cultures. Salvin (1949) reported the yeast form grows well with several amino acids such as glycine, or glutamic acid as the sole carbon and nitrogen sources. Most reports in the literature including Gilardi and Laffer (1962) found no vitamin requirements for either form.

In this study the basic salts medium of Gilardi and Laffer (1962) was used to determine the nutritional requirements of the mutant strains and strains of B. dermatitidis. The vitamin requirements were investigated for several of the mutants by the addition of thiamine and biotin to the basal medium containing asparagine (aBM). With either biotin or thiamine in the medium no increased growth occurred for mutant strains or for the parent St. Joseph strain at 24 C. This corresponded with the results reported by Gilardi and Laffer (1962).

When two additional amino acids, glycine and tyrosine, were put into the basal medium with asparagine (aBM), about a 10 fold increase in mycelial growth occurred at 24 C. Salvin (1949), and Gilardi and Laffer (1962) have reported glycine as one of the amino acids that gave good growth, while tyrosine was not readily utilized. With one amino

acid, asparagine, added to the basal medium at 24 C, there was less growth for each strain. This indicated a combination of amino acids gave better growth or limiting factors were present in the media for growth of the organisms. A more complete medium was needed for enzyme studies as vigorous colony growth was necessary.

The media reported by Gilardi and Laffer (1962), Salvin (1949), Levine and Ordal (1946), and Beneke et al. (1969) known as CYPG, were used to compare dry weight of growth at 24 and 37 C for the two strains, St. Joseph and Ga-1, after 15 days incubation. The differences in the two strains with respect to growth were greater on CYPG medium, at 24 C and 37 C, than on the other media. This medium was selected for growth of the mutants for enzyme studies and other investigations.

A marked difference was observed in viability of the yeast cells when the yeast phase was maintained in liquid culture on the basal medium of Gilardi and Laffer (1962). After two or more weeks it was discovered that the transfers of stock cultures were not viable. When either the basal medium plus asparagine, with proline added or the CYPG medium was used, the viability of the yeast cells remained high. Asparagine or a similar factor appears important for viability of the cells when cultured for a 24-day period at 37 C, while a low cell viability occurred when glycine, tyrosine, or proline were substituted. However, these strains were not able to utilize glycine, and proline as readily as was reported by Salvin (1949).

In addition to a wide variation in virulence of the mutant strains, to ones less virulent than the Ga-1 strain considered to be avirulent by DiSalvo and Denton (1963), the other eleven pathogenic strains of B. dermatitidis showed variation but not as much. Reports in the literature indicate other pathogenic fungi have shown similar variation in virulence in different strains when injected into mice. Salvin (1952) reported that four strains of Candida albicans showed variation of virulence in mice. This variation in virulence in three strains of Cryptococcus neoformans was reported by Hansenclever and Mitchell (1960).

The total lipids of the yeast cells for 4 strains, St. Joseph, Oklahoma, Van Camp, and Duke, were determined and the 50% kill in mice was determined. Two strains with a lower lipid content had a higher 50% kill than the two strains with a higher lipid content. This was a variation in results when compared to those reported by DiSalvo and Denton (1963). They suggested the higher lipid fractions of B. dermatitidis have a possible relationship to virulence in mice.

Virulence in the mutant strains were compared with other strains of B. dermatitidis by intraperitoneal injection of the yeast phase cells into mice. The mutants M-26, M-25, and M-37 offer good evidence that the virulence in B. dermatitidis was modified after mutation. There were four of the 26 mutants that had up to a 100 fold reduction in virulence of the organism when injected intraperitoneally

into mice as determined by 50% kill. Some of the other mutants were less virulent than the parent strain of B. dermatitidis yet were more virulent than the four strains considered to be low in virulence, strains M-25, M-26, M-37, and M-40. When Ga-1 strain, reported by DiSalvo and Denton (1963) to be avirulent, was compared with mutant strain M-37, the latter was less virulent when a higher concentration of cells was injected into mice. At a concentration of 1×10^7 cells per ml injected into mice, there was no reaction at the end of 90 days for both strains. However, if the concentration were increased to 1×10^8, the 50% kill in mice occurred in 21 days for Ga-1 strain and in 78 days for the M-37 strain. This indicated less virulence for the M-37 strain. More strains need to be examined to determine if this variation continues to differ or there may be a correlation in some cases with results reported by DiSalvo and Denton.

The report by Taylor (1964) indicated a possible toxin is present in B. dermatitidis that caused the death of mice. A similar reaction was observed when trypsin-treated cells showed a toxic reaction similar to that described by Taylor as indicated by the time of death in mice. The toxin level was too low in concentration to be detected. Repeated intraperitoneal injections of heat-killed B. dermatitidis were reported by Baker (1942a) to be toxic in mice and often lethal. The repeated injection of heat-killed cells was not done in this investigation. The more rapid rate of kill

in mice for strains used confirms the indication of a possible toxin present in B. dermatitidis that caused the death in mice.

Salvin (1952) reported a toxin from dead cells of B. dermatitidis. He found that the acetone or heat-killed cells caused death in 48 hours of all the mice. Repetition of this procedure using heat or acetone killed cells in mice failed to confirm a toxin in the animals even after 60 days. The control mice inoculated with 2.8×10^6 live cells had a 50% kill of 17 days. There may have been less toxin in the strain selected or the laboratory animals may have had a higher tolerance for any toxin present. However, in the living cells, excessive number of cells inoculated intraperitoneally into mice, indicate a toxin present, confirming previous reports. The trypsin treated cells also confirmed the possibility of a toxin being present. However, the exact nature of the toxin effect has not been determined.

Alkaline and Acid Phosphatase Activities

The determination of the alkaline and acid phosphatase activities on solid media for the mutants and strains of B. dermatitidis followed the method described by Beneke et al. (1969) for detection of alkaline and acid phosphatases in media by the use of para-nitrophenyl substrates. Under these conditions the extracellular acid phosphatase was generally

higher in the yeast phase at 37 C than the mycelial phase for all of the mutants. In contrast, when 8 of the mutants and 6 strains of B. dermatitidis were grown in liquid culture at 37 C, the alkaline phosphatase activity was higher. The alkaline phosphatase activity was high and more uniform for the strains of the mutants but variable in the 6 strains of B. dermatitidis. A possible reason for a variation at 37 C and 15 day incubation period is that some strains may reach peak growth in the yeast phase in 10 days while other may require a longer period.

The peak of yeast-cell growth is about 8 days, while the alkaline phosphatase is highest in the filtrate of the St. Joseph strain in about 10 days. The level of the alkaline phosphatase activity then shows a rapid decrease up to day 30. The peak of growth for the mycelial phase is about 10 to 12 days while the peak of enzyme activity is about 20 days, or at a later period for the 24 C cultures. The relationship of virulence and the level of phosphatases was checked by intraperitoneal injection of the yeast cells into mice. No correlation was shown when strain M-26 and the St. Joseph strain were compared. The virulence is about 100 times greater with the St. Joseph strain than with the mutant M-26. However, the enzyme levels for alkaline and acid phosphatase are about the same in the data for these experiments.

The gel electrophoresis assays of the parent and one of the less virulent mutants, M-26, following a modification of the Davis method (1951), gave no indication of any pronounced differences in the protein patterns. Minor differences were observed in a single chromatogram but this difference was never significant. When the protein gels were stained for alkaline phosphatase activity, the activity was much higher at 24 than 37 C. The sites of the alkaline phosphatase activity in the strains examined were identical in the gels. This result corresponds with the results reported by Stipes (1970). He found that the sites of alkaline phosphatase activity were identical for each isolate within each species of Ceratocystis fagacearum, C. coerulescens, and other fungi. Additional protein patterns would need to be made on the various mutant strains to see if any detectable differences can be identified when compared with the parent strain.

In order to determine if alkaline and acid phosphatase were membrane bound, the KCl extraction procedure of Weimberg (1969) was followed. It was found that better than 10 times as much alkaline phosphatase was present inside of the cells as was released into the growth medium. The acid phosphatase was 4 to 5 times as great inside the cells as it was in the growth medium. This result indicates the alkaline and acid phosphatases are not released from the cell surface of B. dermatitidis. Weimberg and Orton (1965) reported that 80% of the acid phosphatase was released from

the cell surface of Saccharomyces mellis. These two organisms show a different site for the location of the enzyme.

In order to determine if alkaline and acid phosphatase activities are particle bound, the cell extract was ultracentrifuged at 105,000 X G for 2 hours. Only a very minor drop in the enzyme activity occurred during this treatment, indicating the enzymes were not particle bound. In contrast, Wood and Tristram (1970) reported that Bacillus subtilis was particle bound.

The M-36 strain from the B. dermatitidis St. Joseph strain and the M-26 strain are two of the mutants that should be investigated further. The former remains in the yeast phase at both 24 and 37 C, with less virulence than the parent, while the latter strain is nearly 100-fold less virulent. These strains would be suitable for irradiation with mutigenic agents for the development of an avirulent strain. Such a strain would be useful in a viable vaccine study in animals. Hill and Marcus (1959) found that the inefficacy of the yeast phase vaccine with killed organisms was in part related to the dose used.

Another method of selection for less virulent strains or an avirulent strain would be by crossing some of the mutants to develop cleistothecia. Selection of ascospores from these crosses could lead to development of new strains with a less virulent or an avirulent characteristic. A small number of monoascospore cultures from crosses of B. dermatitidis have shown wide variation in pathogenicity when

inoculated into mice according to McDonough and Lewis (1968). The development of avirulent strains would be a valuable tool for future investigations.

SUMMARY

1. Mutants of Blastomyces dermatitidis, St. Joseph strain were developed by ultraviolet irradiation from yeast-phase cells. Twenty-six of the 45 isolates were selected for study of nutritional requirements, enzyme production and virulence in mice.

2. A mutant strain M-36 was developed which remained in the yeast phase at both 24 and 37 C.

3. The mutants M-25, M-26, and M-37 were found to be more than 100 times less virulent than St. Joseph strain.

4. The St. Joseph and Ga-1 strains, when grown in Gilardi and Laffer's basal medium plus asparagine, had good growth in the yeast phase only. When proline was added both the mycelial and yeast phase showed an increased amount of growth.

5. The vitamins, biotin and thiamine, were not required for the growth of Blastomyces dermatitidis.

6. The addition of asparagine was necessary for Gilardi and Laffer's basal medium in order to maintain viable

cultures for more than 5 to 8 days in the yeast phase at 37 C. Glycine, tyrosine and proline could not be substituted for asparagine.

7. Since the organism did not grow as well on the basal salts medium with asparagine, glycine and tyrosine as on the enriched medium, the latter was used for enzyme studies.

8. Trypsin-treated yeast cells at 40 mg/mouse had a higher virulence on mice than the untreated cells.

9. There was a variation in the amount of lipid content in the yeast cells with no apparent correlation to virulence in strains.

10. The alkaline phosphatase activity was generally higher than the acid phosphatase in the liquid medium at 37 C, and at 24 C. The extracellular alkaline and acid phosphatase activities remained low at 37 C but higher at 24 C over a 30-day period. The intracellular alkaline and acid phosphatases were usually much higher than the extracellular enzymes for both phases.

11. The alkaline and acid phosphatases are not particle bound. Both enzymes were not easily removed from the cell membrane.

12. No correlation could be made between virulence in mice and the alkaline or acid phosphatase level.

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