EXTRACELLULAR ENZYME ASSAYS OF BLASTOMYCES DERMATITIDIS AND HeLa CELLS IN COMBINED AND SEPARATE POPULATIONS

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ABSTRACT

EXTRACELLULAR ENZYME ASSAYS OF *BLASTOMYCES DERMATITIDIS* AND HeLa CELLS IN COMBINED AND SEPARATE POPULATIONS

By

June Dale Hillelson

The production of extracellular enzymes by *Blastomyces dermatitidis* Gilchrist and Stokes, 1898, was investigated while the organism was growing in an environment more nearly comparable to that which supports the infecting yeast.

HeLa cells, yeast cells, or a combination of these two cell types were grown in spent (aged 3 days and 11 days) and fresh tissue culture media enriched with 10% fetal calf serum. Exogenous alkaline phosphatase was also placed into the spent material, in a separate experiment. At 12 hour intervals for 60 hours, samples to be assayed were removed from each of the flasks. In another experiment, HeLa cells were combined with yeast cells in order to observe the total number of intracellular fungal organisms.

Paranitrophenol (PNP) derivatives were chosen as substrates for 7 of the 11 enzymes studied. The remaining enzymes were assayed by the Sigma methods.

When combining *B. dermatitidis* yeast cells with HeLa cells, the two cell types might be stimulated to produce a greater quantity of exoenzyme than either of the cell types alone. The overall significance lies not so much in the production of the enzyme, but in its change in activity. The data were based on a comparison of the results of enzyme activity in the medium of the HeLa plus Fungus combination flask to the activity in the medium of each of the separate flasks, and by comparing enzyme activity in the medium of the separate flasks to their respective controls.

When utilizing spent tissue culture medium, the data showed immediate increases and/or gradual increases in levels of activity for β-D-glucosidase, N-acetyl-β-glucosaminidase, glutamic-oxalacetic transaminase (GOT), and glutamic-pyruvic transaminase (GPT) in the medium of the HeLa flask. The results were similar for GPT in the Fungus flask, and with alkaline phosphatase in the HeLa plus Fungus flask medium. Immediate decreases and/or gradual decreases in levels of activity for alkaline phosphatase, exogenous alkaline phosphatase solution, acid phosphatase, and α-hydroxybutyric dehydrogenase (α-HBD) occurred in the medium of the HeLa flask. Similar results were observed with acid phosphatase and α-HBD in the Fungus flask medium, and with β-D-galactosidase, acid phosphatase, GOT, and GPT in the combination flask medium. HeLa cell lysis (60 hours) in the presence of yeasts produced decreased levels of activity for N-acetyl-β-

glucosaminidase, alkaline phosphatase, and acid phosphatase, but an increase in activity occurred with β-D-galactosidase.

When utilizing fresh tissue culture medium, the data showed immediate increases and/or gradual increases in levels of activity for acid phosphatase, GOT, GPT, and α-HBD in the HeLa flask medium. Similar results occurred with GOT in the Fungus flask medium. The combination flask medium at the zero hour demonstrated increased levels of activity for N-acetyl-β-glucosaminidase, alkaline phosphatase, and acid phosphatase. Glutamic-oxalacetic transaminase was increased in the combination flask medium through 48 hours. Immediate decreases and/or gradual decreases in the levels of activity of β-D-glucosidase, N-acetyl-β-glucosaminidase, acid phosphatase, and GPT were observed in the combination flask medium. Similar results occurred with α-HBD in the Fungus flask medium. HeLa cell lysis in the presence of yeasts produced decreased levels of β-D-glucosidase, N-acetyl-β-glucosaminidase, β-D-galactosidase, and alkaline phosphatase.

Inhibitors produced by the yeasts or HeLa cells, and enzyme half-life, as well as production or destruction of the enzymes may have produced the above changes in enzyme activities.

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A THESIS

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To my parents

and

Blastomyces dermatitidis

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INTRODUCTION

The investigation of extracellular enzyme production by any organism is enhanced by cellular enzyme localization. In studies of various fungal cells other than *Blastomyces dermatitidis*, vacuoles have been the major site of cytochemical localization of acid hydrolases (2,37). Both acid and alkaline phosphatases have been reported intracellularly. In *Candida albicans*, acid phosphatase was observed during different phases of cell growth (58). The activity increased up to 15-18 hours and then decreased. Sites of acid phosphatase activity were found in intracellular granules similar to lysosomes or in cytoplasmic vacuoles. In *Schizophyllum commune*, acid phosphatase activity was found to be associated with vacuoles, endoplasmic reticulum, lipid bodies, small mitochondrial granules, and the nuclear envelope (70).

Acid phosphatase activity has also been demonstrated in fungi during lytic processes, such as the release of ascospores from asci (105) and during the autolysis of fruiting bodies (44).

Yeast protoplasts have been demonstrated to contain and have released acid phosphatase. Whether or not the enzyme was located within the plasmalemma or outside the protoplast was studied by cytochemical means, in order to

gain more insight into the process of secretion of the exoenzyme. In *Saccharomyces cerevisiae*, acid phosphatase was found localized over the cell wall of both the mother cell and its bud, and over the forming septum (7). Adjacent cell wall vesicles also showed acid phosphatase activity.

Prior to cytochemical studies, evidence for concluding that certain phosphatase enzymes were on the cell surface was that phosphorylated substrates considered incapable of penetrating the cell membrane were hydrolyzed by intact cells. Later scientists began to isolate cell walls that were enzymatically active or converted yeast cells into protoplasts and showed that the enzyme was released from the cell as the cell wall was digested.

Support for the study of extracellular enzymes is evidence which relates the importance of specific enzyme to the development of the organism. Extracellular enzymes cleaving β-glucosidic linkages have been suggested to play a role in the maturation of certain fungi (104). The significance of these enzymes cleaving β-glucosidic linkages may be that most of the higher fungi have cell walls rich in the various β-glucans. These enzymes are probably involved in cell wall alteration during various morphogenetic events. Other studies with this enzyme deal with the role of β-glucosidase in the metabolism of cellulose and β-glucosides (25). Biochemical and genetic studies have described certain enzymes whose expression is closely related to the control of progress through the developmental

phase of the life cycle of *Dictyostelium discoideum* (21). These studies indicated that the expression of β-glucosidase was directly related to the control of development. It can further be described as stage specific or developmentally controlled.

An increase in both α-D-galactosidase and β-D-glucosidase activity as the basidiocarp of *Pleurotus ostreatus* reaches maturity suggests that these enzymes could be related to the fruiting process (55). Higher fungi may be expected to have α-galactosidase in order to utilize the α-galactosides present in plant materials.

The exact role of acid and alkaline phosphatase in metabolism is unknown. Acid phosphatase activity is associated with the meiotic process in *Calvatia cyathiformis* because of the increased activity found in meiotic tissue, as opposed to that found in post-meiotic tissue (12). The loss of alkaline phosphatase activity, on the other hand, is correlated with the inability of certain mutants of *Aspergillus nidulans* lacking alkaline phosphatase to cleave organic phosphates (67).

Several enzymes have been localized and have been shown to be important in the development of several fungi, but relatively few have been demonstrated extracellularly. This is particularly true for those fungi causing systemic disease. Beneke et al. (9) investigated the presence of several extracellular enzymes of *B. dermatitidis*. The yeast phase (37 C) was reported to produce alkaline and acid phosphatase when grown in both liquid and solid

casein-peptone-yeast extract-glucose (CPYG) agar. Unpublished data showed that lower phosphatase levels were produced when the yeast phase was grown in brain-heart-infusion medium. No α- or β-glucosidases, α- or β-galactosidases, N-acetylglucosaminidases, or fatty acid esterases were detected.

The objective of this research is to investigate the production of exoenzymes by *B. dermatitidis*, and to study the enzyme activities in an environment more nearly comparable to that which supports the infecting yeast.

Blastomyces dermatitidis can be maintained more readily in the yeast phase in tissue culture medium with HeLa cells than in artificial medium. By placing yeast cells together with HeLa cells, both in spent and in fresh tissue culture media, one might expect that the two cell types in combination will be stimulated to produce a greater amount of exoenzyme than either of the individual cell types alone. Exogenous enzyme will also be placed into the spent medium experiment, in order to observe its activity over a period of time. Finally, parasitism of the yeast cells by the HeLa cells will be investigated.

LITERATURE SURVEY

Blastomyces dermatitidis Gilchrist and Stokes, 1898, the imperfect stage of an Ascomycete, *Ajellomyces dermatitidis* McDonough and Lewis, 1968, is the causative agent of North American blastomycosis. It is a dimorphic fungus, growing in tissues in culture at 37 C as a round or oval, 8-15 μ thick-walled budding yeast and on conventional media (25 C) as mycelium giving rise to pear-shaped or exogenous spores. Conversion from mycelial to yeast growth, and vice versa, is easily accomplished *in vitro* by varying the temperature. Ricketts (73) and Hamburger (31) were among the first to observe *in vitro* fungal growth and indicated temperature as an influential factor in differentiating between the two morphological types. This finding has since been confirmed by Michelson (56), DeMonbreun (18), and Levine and Ordal (52). Salvin (83) concluded that no specific carbohydrate, amino acid, or other "growth substance" was implicated as an essential factor for yeast-like growth. The organism grew best in a medium containing only serine or hydroxyproline as sole amino acids, or in a medium containing twenty different amino acids. The amount of growth produced, however, did vary according to the medium.

The fungus of North American blastomycosis is found mostly in the central and southeastern portions of the United States and, less frequently, in Canada and Africa. Defining the geographic distribution of blastomycosis is difficult due to the lack of availability of an acceptable method to detect infection, and the inability to recover the etiological agent from its saprophytic environment. The natural habitat of *B. dermatitidis* remains problematic. Since infections appear to be acquired by inhalation of spores, the organism is considered to be a soil saprophyte. However, only a few attempts at isolating the organism from soil in endemic areas have been successful. Spores have been isolated from the soil in Lexington, Kentucky, and in Georgia (19,20). *Blastomyces dermatitidis* has been cultivated in the laboratory on sterilized soil (6,27), but the basic problem with soil isolation was that repeated sampling for the organism from previously positive locations turned out to be negative. Dogs, along with other animals, have frequently acquired this disease, but are not known to transmit the infection to man.

North American blastomycosis, or Gilchrist's disease, named for the man who first reported the skin disease in 1894 (76), is divided into three clinical patterns of disease, cutaneous blastomycosis, pulmonary blastomycosis, and systemic dissemination (8). Cutaneous blastomycosis is acquired by spore inhalation and spread to the skin, or by implantation of spores into broken skin. The lesions occur on the more exposed parts of the body, namely hands,

wrists, face, feet, and ankles. The initial lesion may spread slowly during the course of the disease, which may be for months or years. This may be followed by satellite lesions occurring in more distant areas.

Pulmonary blastomycosis, developing from spore inhalation, is manifested by miliary abscesses in the lung. This is usually accompanied by granulomatous reactions. The yeast cells are visible within the abscesses and granulomata. From the lungs, infection can spread hematogenously to establish focal lesions in skin, bones, prostate and other viscera, except for the gastrointestinal tract, to produce systemic blastomycosis.

The lesions of North American blastomycosis are not unique to man. Bowen and Wolbach (11) inoculated four mice intraperitoneally and observed abdominal and pulmonary lesions. They described the pathologic process as "filling of the alveoli with large cells with little other reaction." After having injected the yeast form intraperitoneally, DeMonbreun (18) found that "each of six mice died in from three to five weeks after inoculation, and numerous small abscesses containing the fungus cells were found in the lungs, liver, spleen and kidneys." In mice inoculated intraperitoneally with yeast by Bergstrom et al. (10), lesions developed in five weeks, but the animals did not die until ten weeks following inoculation. Davis (16) observed that the organisms were taken up by leukocytes and macrophages following injection into the peritoneal cavity of guinea pigs.

Salfelder (82) found that subcutaneous injections of the yeast form of *B. dermatitidis* produced a non-ulcerative active skin lesion in animals, which healed without dissemination.

During their laboratory induced disease study, Ebert and Jones (24) found that 10^5 yeast cells injected intraperitoneally were sufficient to produce blastomycosis in dogs. Treatment with amphotericin B, the drug of choice, prevented the death of some of the injected animals.

Baker (5), working with yeast cells, injected mice intraperitoneally with living *B. dermatitidis*, killed suspensions of the organism, and a phosphatide fraction of this fungus. He found that repeated injections of killed yeasts were toxic and often lethal for mice. The lesions, in mice receiving the live yeast cells, were mostly composed of organisms, as opposed to other infectious diseases in which the lesions primarily consist of the reacting host cells. The phosphatide fraction brought about a monocytic response, and was therefore not implicated in the necrotizing effect produced by the living organisms. Baker did not continue studying the phosphatide fraction because he was particularly interested in the suppurative response.

Peck and Hauser (65) isolated minute amounts of a phospholipid-containing carbohydrate fraction from the yeast form of *B. dermatitidis*. Later, Peck (64) found that this constituent was associated only with pathogenic fungi.

Prior to the work of DiSalvo and Denton (22) in 1963, relatively few attempts had been made to determine cellular constituents responsible for the virulence of *B. dermatitidis*. These authors looked at four strains of *B. dermatitidis* and observed a greater abundance of lipid in the more virulent of the four strains.

Having demonstrated in 1972 (14) that two strains of *B. dermatitidis* with different virulence for mice also differed in the amount of bound phospholipid present in the cell wall, Cox et al. (15) later attempted to clarify certain aspects of the pathogenesis of blastomycosis by looking at host resistance and the factors relating to the virulence of the infecting agent. Mice were inoculated with cell-wall fractions from the two above strains, to compare the histological responses and the differences in virulence. The same pathologic response resulted when either whole cells or just cell walls were injected. Infarct-like tissue response was common to both groups, and the necrosis seemed limited to the areas of accumulated inocula. From this, the authors suggested that the cell walls might possess endotoxin activity, but made no conclusions as to the relationship between toxicity and necrotic lesions.

In 1952, Salvin (84) demonstrated specific endotoxin by injecting acetone-dried yeast cells of *B. dermatitidis* and tubercle bacilli intraperitoneally into mice. Death occurred in about 80% of the mice in 48 hours when components from 80 mg of acetone-dried cells were injected.

Taylor (98) found that a similar toxin was released when *B. dermatitidis* yeast cells were treated with trypsin or with 1% HCl. Lethality was enhanced when a suspension prepared from disrupted yeast cells, containing the specific endotoxin, was injected intraperitoneally into mice previously inoculated with Newcastle disease virus (43).

The specific factors implicated in fungal infection have been less investigated than those mechanisms in bacteria. Attempting to correlate virulence with an endotoxin is as much an enigma as trying to correlate virulence with an enzyme or a group of enzymes. Enzyme production alone by an organism does not seem sufficient for pathogenesis. For example, *Trichophyton evolceanui* and *T. indicum* are two non-pathogens which produce elastase (38). Rippon and Varadi (78), on the other hand, demonstrated elastase production by a relatively old culture of *Streptomyces madurae*, a pathogen, and no production of this enzyme by any of the other actinomycetes tested.

Purnell and Martin (67) were the first to associate alkaline phosphatase activity with virulence in any pathogenic organism. They found that certain mutants of *Aspergillus nidulans*, lacking alkaline phosphatase and unable to grow on medium with beta-glycerophosphate, had a decreased virulence for mice. Mutants without alkaline phosphatase that were able to grow on the medium were virulent. Also, a mutant without alkaline phosphatase which could grow on the beta-glycerophosphate medium was

virulent. The authors reported a definite decrease in virulence of certain strains associated with the presence of specific mutations affecting alkaline phosphatase activity. Decreased virulence in those strains without alkaline phosphatase was associated with an inability to split certain organic phosphates.

Montes and Wilborn (58) investigated *Candida albicans* cytochemically throughout the logarithmic and stationary growth phases. Intracellular activity increased up to 15-18 hours of growth and then began to drop. Acid phosphatase activity was located in small intracellular granules or in yeast vacuoles. The authors suggest that this enzyme activity correlates directly with fungal virulence, but this relationship needs to be clarified through further experimentation.

In *C. albicans*, toxic activity has been reported to be associated with hydrolytic enzyme activity and the presence of an intracellular alkaline phosphatase by Kurup (48), a leucine aminopeptidase in cell extracts by Kim et al. (46), an excreted peptidase by Staib (92), and a plasma coagulase by Zaikina and Elinov (108). In media containing protein as the nitrogen source, proteolytic enzymes are excreted by certain strains of *C. albicans* (72). Only the proteolyzing strains caused severe infection when injected into mice.

Intracellular Enzymes

Much of the fungal enzyme work to date has been done using intracellular enzymes produced by fungi other than *B. dermatitidis*.

Glucanase

Glucanase production by *Histoplasma capsulatum* and *B. dermatitidis* was investigated by Davis and Domer (17) with the intention of locating autolytic enzymes capable of degrading cell wall carbohydrates from these two organisms. Glucanases were found in cellular extracts and in culture filtrates from the yeast and mycelial phases of both organisms. The fungal enzymes appreciably hydrolyzed only the glycoprotein fraction of the cell walls. It was suggested that a relationship between cell wall growth and these autolytic enzymes may exist.

Malate Dehydrogenase

Isoenzymes of malate dehydrogenase (80) were studied in cell-free extracts, mitochondrial fractions, and cytoplasmic portions of both the mycelial and yeast phases of *B. dermatitidis*. Through the use of electrophoresis on polyacrylamide gels, isoenzyme patterns of both fungal phases were apparent in several bands. The mitochondrial fraction of the yeast phase demonstrated two bands, whereas five bands were present in the cytoplasmic portion of the same phase.

Galactosidases

Intracellular β-D-galactosidase activity in single yeast cells of *Saccharomyces lactis* taken from a random population was measured (106) using a fluorogenic assay. Photographs were taken to establish specific stages of the growth cycle of each yeast cell. Similar enzyme levels were found in cells without buds and in cells with intermediate-size buds. Mature cells, or nearly mature cells, possessed approximately double the level of this enzyme.

Alpha-galactosidase received attention from Arnaud et al. (3). They grew *Penicillium duponti*, a thermophilic fungus, in medium containing either raffinose or stachyose. This particular enzyme was selected because of its ability to hydrolyze raffinose in beet molasses and to hydrolyze oligosaccharides in vegetable seeds. Their experiment showed that α-galactosidase from *P. duponti* exhibited thermostability characteristics different from other fungal α-galactosidases.

Phosphatases

In 1974 (61) acid phosphatase was used to investigate the relationship of enzyme levels in the identification and the taxonomy of pathogenic species of *Candida*.

It is known that nonspecific acid phosphatase is localized on the outer surface of the cell membrane in *S. cerevisiae* (86,96). Ueda and Oshima (100) described a recessive constitutive mutant, phoT, with decreased

ability to assimilate inorganic phosphate (Pi). Recessive constitutive mutants, phoR and phoU, were unable to accumulate Pi.

Komano (47) related the culture age of *A. niger* to the formation of multiple forms of acid and alkaline phosphatases by first extracting the mycelium, separating and purifying into four fractions, and then observing the changes in activity of these phosphatases in peptone medium during growth.

Extracellular Enzymes

The pathogenic significance of enzymes, if any, depend partly on whether or not they are excreted from the organism or if they are associated with the fungal cell surface so they may attack host substrates.

Phosphatases

Differentiating between the products of secretion and autolysis is often difficult. By using alkaline phosphatase as a cell marker, proteases of *Microsporum canis* (a dermatophyte) were determined to be truly extracellular (63). Since the autolytic marker enzyme was present in minute amounts when the proteases appeared, the process was thought to be true secretion.

Beneke et al. (9) reported the production of extracellular acid and alkaline phosphatases by species of *B. dermatitidis* in a single culture medium. The yeast and mycelial phases were shown to secrete different amounts of enzyme into the culture medium. Enzymes displaying no

activity in the culture medium were: β-glucosidase, α- and β-galactosidases, N-acetylglucosaminidase, and butyrate, palmitate, acetate, laurylate, and stearate esterases.

Urease

Urease is particularly interesting because it was the first enzyme to be isolated in crystalline form and the first example of an enzyme shown to contain sulfhydryl groups (95). Shibata (87) found urease in *A. niger* mycelium, whereas Kiesel (45) found both urea and urease in the same organism.

Much later, Rosenthal (79) incorporated a specific substrate into the medium, in order to enhance specific identification of pathogenic fungi. An application of this is seen today in the differentiation of two similar species of dermatophytes. *Trichophyton rubrum* does not hydrolyze the urea in 7 days, as opposed to *T. mentagrophytes*, which does hydrolyze the substrate.

In 1962, urease was demonstrated as a specific substrate in *B. dermatitidis* (99). In growing cultures with urea as the sole nitrogen source, and in cell extracts, both the mycelial and yeast phases demonstrated a constitutive urease activity. This activity was shown to be both intracellular and extracellular.

Uricase

In 1962, Taylor (97) found uricase in the mycelial phase of *B. dermatitidis*, but not in the yeast phase of

the organism. Under alkaline conditions (pH 9.0), this activity was inhibited, but in the mycelial form the organism continued to grow well and to utilize uric acid acid at pH 6.5, 7.0, and 8.0, at 25 C.

Glucanases and Xylosidases

Within the last two years, extracellular glucanases have been studied extensively in fungi. Beta-glucanases have been produced and separated in asynchronous cultures of *Cryptococcus albidus* var. *aerius* (59). In *Schizosaccharomyces japonicus* var. *versatilis*, an exo-beta-glucanase was isolated (28). Eriksson and Pettersson (1,26,94) worked extensively with exo- and endo-1,4-beta-glucanases used by *Sporotrichum pulverulentum* for cellulose breakdown. In 1975, they began by purifying, characterizing, and separating five endo-1,4-beta-glucanases (94). In their next publication, the activities of the five enzymes towards carboxymethylcellulose were compared (1). Following this, purification and characterization of an exo-1,4-beta-glucanase were performed (26). Beta-xylosidases were also detected by Notario et al. (60) in *Cryptococcus albidus* var. *aerius*, provided the medium contained glucose as the sole carbon source. Friebe and Holldorf (29) worked on the inactivation of 1,3-beta-glucanase activity in the Basidiomycete species QM806.

Glucosidases

A large molecular weight glucosidase of *A. fumigatus* was purified and, by polyacrylamide gel electrophoresis

followed by carbohydrate and protein staining, was shown to be a glycoprotein (81).

Wilson and Niederpruem (102) examined the regulation and nature of enzymes cleaving beta-glucosidic linkages in *Schizophyllum commune* in order to relate these particular enzymes to mycelial growth and basidiospore germination. They found the enzymes to be present in low amounts during growth and germination.

Galacturonanase

Through salting-out and two different types of chromatography procedures, an exo-D-galacturonanase was purified from a culture filtrate of *A. niger* (32). This enzyme not only catalyzes the degradation of D-galacturonans by terminal action but also acts on other substrates of medium and high molecular weights with the same terminal ends.

Ribonuclease

Cryptococcus laurentii and its haploid Basidiomycete relative, *Tremella foliacea*, exhibited ribonuclease production in liquid medium (13), which was completely repressed in both fungi by the addition of orthophosphate. These organisms have a close affinity to the Basidiomycetes and certain Ascomycetes which were shown to have extracellular ribonuclease activity. Other Ascomycetes tested did not have this activity. These results suggested a close relationship between the two previously mentioned fungi.

Collagenase

An extracellular collagenase from *T. schoenleinii* was observed (74) to be different from bacterial collagenase in having a smaller molecular weight and requiring an acid pH. This fungal enzyme proved to be inhibited by ethylenediaminetetraacetic acid (EDTA) as opposed to bacterial enzymes. The activity of the bacterial enzyme can be restored by magnesium and calcium.

Multiple Enzymes

In 1969, Rippon and Garber (77) investigated dermatophyte pathogenicity as a function of mating type and related enzymes. Enzyme profiles of several dermatophytes and *B. dermatitidis* exhibiting enzymatic differences in sexual stages were demonstrated in 1971 (75). In the yeast phase of *B. dermatitidis*, the plus mating type produced elastase, urease, and hydrolyzed casein, while the minus mating type produced urease, and hydrolyzed casein, but no elastase. One mycelial strain (M784) tested produced alkaline phosphatase, acid phosphatase, elastase, leucine amino peptidase, urease, and hydrolyzed casein. The other mycelial strain (M788) produced gelatinase, alkaline phosphatase, acid phosphatase, leucine amino peptidase, urease, and also hydrolyzed casein.

Tissue Culture and Fungi

Tissue culture techniques have been employed to a limited extent in the study of pathogenic diphasic fungi. Friedheim and Baroni (30) were the first to look at the

effects of fungi on cultured animal cells *in vitro*. By inoculating guinea pig fibroblast cultures and mouse embryonic tissue, and splenic leukocytes of mice and guinea pigs with *Nocardia asteroides*, they observed disintegration of the fibroblasts and leukocytes. They attributed this disintegration to the exhaustion of nutrients required by the animal tissue cells to the rapid growth of the fungus.

Pathological information concerning work on this inoculation of chicken and rat fibroblasts with *B. dermatitidis*, *Coccidioides immitis*, *H. capsulatum*, and *M. lanosum* was recorded by Duque (23). *Blastomyces dermatitidis* was shown to have no effect in his tissue culture system.

Randall and McVikar (93) observed the development of yeast cells of *H. capsulatum* in fibroblast cells of tissue cultures prepared from horse placental tissue and chick embryos. *Histoplasma capsulatum* has also been cultured in human tissues (68), in HeLa cells (51), and in Earle's L-strain mouse cells (69). Upon subsequent inoculation of mice with *H. capsulatum*, Hill and Marcus (35) demonstrated that cultured mononuclear cells from the animals were able to restrict the growth of this yeast.

Larsh et al. (49) demonstrated the value of HeLa cells in culture for converting the mycelial phases of *B. dermatitidis*, *H. capsulatum*, *C. immitis*, and *Sporothrix schenckii* to their yeast phases. Previously, Lubarsky and Plunkett (53) noted that with some strains of *C. immitis*

tissue phase conversion occurred in animal tissue culture medium alone.

HeLa cell culture techniques were found to be valuable in the laboratory identification of the diphasic fungi (36). Larsh et al. (50) expanded their earlier report to include tissue culture as providing an efficient tool for the evaluation of antifungal drugs.

In 1959, Wang and Schwarz (102) studied phagocytosis of several pathogenic fungi and yeasts by human white blood cells. This report described the general features of phagocytized yeast-like organisms *in vitro*.

Howard and Herndon (42) compared cultured peritoneal exudate cells inoculated with the mycelial phase of *B. dermatitidis* to those inoculated with the mycelial phase of *H. capsulatum*. Both fungi converted to the yeast phase. It was found that *B. dermatitidis* destroyed cell cultures to a degree directly proportional to the number of inoculated fungal cells. Also, the fact that *B. dermatitidis* had a strong tendency to form aggregates during proliferation greatly hindered any study of effective intracellular parasitism. From this work, the authors concluded that *B. dermatitidis* was less suited to animal tissue culture studies involving peritoneal exudates.

In 1964 Howard (39) reported an intracellular generation time of 10.3 $\pm$ 1.5 hr for *H. capsulatum* in mouse histiocytes, and said that this generation time was not affected by the age of the histiocytes. From these earlier data, Howard (40) published findings on the

intracellular growth of five strains of *H. capsulatum* in guinea pig and mouse histiocytes. The intracellular generation time was not changed when yeast cells were exposed to specific antibodies and complement. No differences were noted in cells from immunized and normal animals, with or without previous exposure of the *Histoplasma* cells to complement and hyperimmune sera.

Wagoner et al. (101) undertook a study of conversion to the yeast phase of various *H. capsulatum* isolates in stationary HeLa cell cultures. Conversion took place in Hely or Medium 199, with fresh guinea pig serum. When calf, human, horse, and chicken sera were used, conversion took place to a lesser extent. Mycelium two, four, and eight weeks of age displayed no apparent differences in their ability to convert to the yeast form.

Hempel and Goodman (33) described a convenient method for the positive identification of *H. capsulatum, B. dermatitidis,* and *S. schenckii* whereby the time required for conversion to the yeast phase is considerably shortened. Mycelial phases of each of these organisms were used to inoculate primary cell cultures of guinea pig peritoneal macrophages. These tissue cultures were stained after 24 hours and the characteristic yeast cells were observed microscopically.

The effect of different temperatures (25, 30, 37 C) on the intracellular growth of *H. capsulatum* was questioned by Howard (41). Specifically, data concerning blastospore formation, germ tube development, and yeast-phase growth

within mouse, frog, and fish histiocytes was reported.

In 1967, Stanley and Hurley (93) compared the growth of various species of *Candida* in mouse renal epithelial cell cultures with that of a control yeast, *S. cerevisiae*. Cultures of murine renal epithelial cells were destroyed at different rates by the various species of *Candida* and were partially destroyed by the non-pathogenic control. The rate of destruction was directly proportional to the size of the fungal inoculum. From this, the authors suggested that pathogenic mechanisms are an expression of the effects of fungi on mammalian cells.

MATERIALS AND METHODS

Organisms and Cultivation

Blastomyces dermatitidis

The Oklahoma strain, isolated from a patient in the Oklahoma Medical Center, The University of Oklahoma, Oklahoma City, Oklahoma, was obtained from the laboratory of Dr. E. S. Beneke and Dr. A. L. Rogers, Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan. The mycelial phase, initially grown on Sabouraud's glucose agar, was transferred to brain-heart-infusion agar (BHIA) and placed at 37 C in order to obtain conversion to the yeast phase. The mycelial phase was not investigated. After conversion to the yeast phase on BHIA, the cells were subsequently washed in sterile physiological saline, centrifuged, and placed into Joklik-Modified minimum essential medium (MEM) without phenol red (GIBCO). Sterilized fetal calf serum (GIBCO) was added to obtain a ten percent solution. This stock culture was maintained on a rotator shaker (New Brunswick Scientific Company, model G2), at 200 revolutions per minute. Every ten days the yeast suspension was resuspended in fresh MEM with 10% fetal calf serum, and returned to the shaker.

All tissue culture media refrigerated longer than one month were supplemented with 29.2 mg of L-glutamine (GIBCO) per 100 ml and streptomycin sulfate (Pfizer) at 100 mcg per ml plus penicillin G (Pfizer) at 100 Units per ml prior to use.

Throughout the entire experiment, sterility checks using sheep blood agar and Sabouraud's glucose agar were done whenever the contents of any flask were manually disturbed.

HeLa

Stock cultures of the HeLa (human epithelioid carcinoma), originally from the American Type Culture Collection Cell Repository, were obtained from Wayne Roberts, Clinical Microbiology Laboratory, Michigan State University, East Lansing, Michigan. The serial line in monolayer was aseptically maintained at 37 C in 75 cm^2 plastic Falcon tissue culture flasks. Joklik-Modified MEM without phenol red was used with 10% fetal calf serum for culturing HeLa cells. A humidified mixture of 5-10% CO_2 with air was maintained to adjust the pH to circa 7.2-7.4. The cultures were routinely transferred under a laminar flow hood when the monolayer became dense (about four days). Culture transfer techniques, utilizing Hank's balanced salt solution (pH 7.2) and trypsin-EDTA (GIBCO), described by Merchant, Kahn and Murphy (54), were followed in this investigation.

Experiment I - Spent Medium

Growth curves of *B. dermatitidis* were done in order to ascertain a period of growth referred to as late log phase. Log phase occurred on the second day of growth and continued through the twelfth day. The eleventh day of yeast growth was chosen for investigation as a period during late log phase.

Two Falcon tissue culture flasks, each containing 100 ml of 10^6 fungal cells/ml MEM with 10% fetal calf serum, were placed at 37 C. (All cell counts were performed by placing one drop of a 1:3 dilution of fungal cells in phosphate buffered saline and 0.4% trypan blue vital stain in normal saline (GIBCO) onto a Neubauer hemacytometer.) A control flask, containing all substances except the organism, was placed at 37 C. The three flasks were incubated for 11 days.

On the eighth day, stock HeLa monolayers were transferred. These two tissue culture flasks, each containing 100 ml of 10^5 HeLa cells/ml MEM with 10% fetal calf serum, were placed at 37 C. One control flask with all substances except cells was placed at 37 C. These flasks were kept in the incubator for three days.

On the eleventh day of fungal growth and on the third day of tissue culture growth, the medium from one of the test monolayers was removed and replaced with the contents of one of the test fungal flasks. The five flasks containing spent media (3 day old and 11 day old media) and sera were labeled and tested as: HeLa, Fungus, HeLa plus

Fungus, Fungus Medium Control (11 day old medium with serum), and HeLa Medium Control (3 day old medium with serum).

Immediately before combining the HeLa cells with the *B. dermatitidis* cells, the hydrogen ion concentrations of all five flasks were adjusted to pH 7.2-7.4. The addition of *B. dermatitidis* with its growth medium to a HeLa monolayer marked the zero hour in the first three experiments. At this time, 0.5 ml from each of the test flasks containing cells was removed sterilely for viability counts. Ten milliliters from each of the five flasks were removed and placed into appropriately labeled sterile screw cap tubes. These sampler tubes were then centrifuged for 10 min at 1000 rpm, followed by the removal of the cell-free supernatants and the placement of the tubes onto ice. From these tubes, volumes corresponding to the amount of test material required for each enzyme assay were aliquoted into separate tubes. Depending on the survival conditions of a particular enzyme, the aliquots were either placed on ice for immediate testing, or placed at 4 C or at -20 C for future testing.

At twelve hour intervals after the zero hour, 10 ml samples to be assayed and separate 0.5 ml samples to be counted were removed from each of the five flasks, prior to adjusting the pH. The samples were then centrifuged and aliquoted in the same manner as previously described zero hour samples.

All phases of all experiments were done in duplicate. The experiments were terminated when dead HeLa cells, which incorporated the intense blue color of the vital stain, were lysed. The lysis occurred between 50 and 60 hours.

Experiment II - Fresh Medium

The contents of a stock tissue culture flask adjusted to 100 ml of 2 x 10^8 fungal cells/ml MEM with 10% fetal calf serum were washed in sterile normal saline. Following centrifugation for 10 min at 1000 rpm, 10^6 yeast cells/ml were placed into two tissue culture flasks containing 100 ml of fresh MEM with 10% fetal calf serum.

At the same time, media from two flasks, each containing a three day old monolayer, were discarded. One of these flasks received 100 ml of fresh MEM with 10% fetal calf serum. The other HeLa flask received the contents of one of the above fungal flasks, again in fresh medium.

The three flasks and a single general medium control (without organism of any kind) were incubated at 37 C. The four flasks containing fresh medium and serum were labeled and tested as: HeLa, Fungus, HeLa plus Fungus, and General Medium Control.

The procedures concerning pH, cell counting, sampling, and aliquoting at the zero hour and subsequent 12 hour intervals until 60 hours, were performed as in Experiment I.

Experiment III - Alkaline Phosphatase

Experiment III followed the same basic protocol as Experiment I, with the following modifications. One of the fungal flasks containing 10^6 cells/ml was added to a monolayer tissue culture flask, as in Experiment I. The first modification was at the zero hour, when 5 ml of an alkaline phosphatase solution (500 µg/ml in MEM with 10% fetal calf serum, followed by millipore filtration) were added sterilely to all flasks except the one monolayer medium to be discarded. Each flask before the addition contained 45 ml of experimental material. The final total volume in each of the 5 flasks was 50 ml. All of the flasks, including the media controls, contained 50 µg/ml of alkaline phosphatase.

Another modification was the removal of only 3 ml of test material per 12 hour interval from all of the flasks, since only the alkaline phosphatase assay was to be performed.

Experiment IV - Phagocytosis

Ten milliliters of 1.56×10^5 HeLa cells per ml of MEM, supplemented with 10% fetal calf serum, were transferred to each of 8 sterile plastic petri dishes. Each petri dish contained a 22 x 50 mm coverglass (Sargent). Twenty-four hours later, 10^6 yeast cells were added to each dish. One coverglass was removed, fixed, and stained with Wright's stain at 1, 2, 3, 5, 9, 24, 51 and 77 hours after exposure to the yeasts. In the HeLa cell culture,

the total number of intracellular *B. dermatitidis* bodies per 43X field was counted.

Extracellular Enzyme Assays

Assays

All enzyme assays were monitored colorimetrically using a Bausch and Lomb Spectronic 20 spectrophotometer. Enzyme activity is expressed in µmol per unit time. Readings were made at 30 second intervals. Whenever available, positive controls (Sigma 2-N) were incorporated into the assay procedures.

Alpha-D-glucosidase, β-D-glucosidase, N-acetyl-β-glucosaminidase, α-D-galactosidase, β-D-galactosidase, Alkaline Phosphatase, Acid Phosphatase

To detect the above various extracellular enzymes produced in the culture filtrate, assays were performed utilizing the appropriate chromogenic substrates (Sigma). Free paranitrophenol (PNP) is released (104) as the enzyme reacts with the substrate.

Seven PNP derivatives were chosen as substrates for the above enzymes studied. Assays were performed every 12 hours for 60 hours. The following substrates and buffers were prepared according to the method described by Beneke et al. (9), with modifications made in some of the buffer concentrations: PNP-α-D-glucoside (0.5 mg/ml), PNP-β-D-glucoside (0.5 mg/ml), PNP-N-acetyl-β-glucosaminide (0.15 mg/ml) all in sodium acetate buffer (0.1 M), pH 5.4; PNP-α-D-galactoside and PNP-β-D-galactoside, both 1.0

mg/ml in citrate-phosphate buffer (0.05 M), pH 7.0; PNP-phosphate (1.0 mg/ml) in 0.05 M Tris-HCl buffer, pH 8.6 for alkaline phosphatase. No change was made in the preparation of PNP-phosphate (1.0 mg/ml) in 0.1 M sodium acetate buffer, pH 5.0, for acid phosphatase.

The assay for the hydrolysis of the above substrates was modified from the method of Beneke et al. (9). One-half milliliter of the medium supernatant (enzyme source) was added to 0.8 ml of the substrate solution. The reaction was immediately stopped by the addition of 1.7 ml of 0.5 M Tris buffer (pH 9.8), which develops the yellow color of free PNP. A duplicate set of cuvettes containing the test material plus substrate solutions was incubated for two hours at 37 C prior to the addition of Tris buffer. Enzyme activity was initially determined as a change in absorbance units at 410 nanometers over a 2 hour period during incubation at 37 C.

Standard curves were made by preparing serial dilutions of free PNP in each of the buffers. To obtain a volume of 3 ml, 0.8 ml of the PNP dilution was combined with 2.2 ml Tris buffer. The absorbance at 410 nanometers was graphed against μmol of liberated PNP.

Glutamic-Oxalacetic Transaminase (GOT)

The method used for assaying GOT was that of The Sigma Chemical Company (91). No modifications of the actual method were instituted. The reaction mixture contained 1.0 ml substrate and 0.2 ml test material. One

milliliter of color reagent was added after an incubation period of one hour at 37 C, followed by the addition of 10 ml of 0.4 N NaOH. Media controls were treated as test samples.

As recommended by Sigma, those samples not being tested immediately may be kept at 4 C for at least 2 weeks. The GOT samples from each major experiment were assayed as a group after two days of refrigeration.

Glutamic-oxalacetic transaminase catalyzes the reaction between α-ketoglutarate and L-aspartate which yields glutamate plus oxalacetate. The amount of oxalacetate formed in one hour is determined by the formation of a hydrazone from oxalacetate which was measured at 505 nonometers.

Standard curves were prepared using a standardized calibration solution containing pyruvic acid. One Sigma-Frankel Unit of GOT forms 4.82×10^{-4} μmol of glutamate/min at pH 7.5 and 25 C. Results of GOT activity were expressed in μmol x 10^4/min at pH 7.5 and 25 C.

Glutamic-Pyruvic Transaminase (GPT)

The treatment of the media controls as tests, the reaction mixture, and the storage of samples to be tested as one major group from each experiment were handled as in the GOT assay.

The activity of GPT was measured in the same manner as the GOT. This enzyme catalyzes the reaction between

α-ketoglutarate and alanine forming glutamate plus pyruvic acid.

Alpha-Hydroxybutyric Dehydrogenase (α-HBD)

Alpha-hydroxybutyric dehydrogenase was assayed by the method of The Sigma Chemical Company (89). The enzyme catalyzes the reduction of α-ketobutyric acid to α-hydroxybutyric acid in the presence of NADH. The amount of α-ketobutyric acid remaining after the incubation period is determined by forming its colored dinitrophenylhydrazone in alkaline solution. The activity of α-HBD is inversely proportional to the intensity of this color, measured at 440 nanometers.

No modifications in the actual procedure were made. The reaction mixture was made by mixing 0.5 ml substrate and 0.5 mg NADH with 0.05 test material. One milliliter of color reagent was added after an incubation period of one hour at 37 C, followed by the addition of 5.0 ml of 0.4 N NaOH.

As recommended by Sigma, those samples not being tested immediately may be stored at -25 C for two weeks. The α-HBD samples from each major experiment were assayed as a group after two days at -20 C. Media controls were treated in the same manner as test samples.

Standard curves were prepared using a stock HBD substrate. One Sigma Unit of α-HBD will reduce one millimicromole of α-ketobutyric acid per minute at 25 C.

Results of α-HBD activity were expressed in μmol x 10^4/ min at 25 C.

Lactic Dehydrogenase (LDH)

Attempts were made to detect extracellular LDH. The enzyme was assayed by the method of The Sigma Chemical Company (90).

RESULTS

Experiment I - Spent Medium

Alpha-D-glucosidase, β-D-glucosidase, N-acetyl-β-glucosaminidase, α-D-galactosidase, β-D-galactosidase, Alkaline Phosphatase, Acid Phosphatase

The extracellular activities of the above enzymes in spent tissue culture medium with the exceptions of α-D-glucosidase and α-D-galactosidase are given in Tables 1 through 5. Standard curves, made by preparing serial dilutions of free PNP in each of the buffers, produced a linear curve with absorbance being directly proportional to μmol PNP.

In the first experiment, a change in absorbance from zero to two hours yielded no measurable α-D-glucosidase or α-D-galactosidase activity in any of the flasks throughout the 60 hour testing period.

The media of the flasks with the Fungus and the Fungus Medium Control demonstrated no enzyme activity in either of the glucosidase assays (Table 1). The HeLa plus Fungus medium showed no β-D-glucosidase activity. From the zero hour to the 60th hour, the HeLa Medium Control did not change in β-D-glucosidase activity. The HeLa medium showed slightly increasing levels of β-D-glucosidase activity with time.

Table 1. Extracellular β-D-glucosidase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	48	48	51	54.5	54.5	54.5
Fungus	0	0	0	0	0	0
HeLa plus Fungus	0	0	0	0	0	0
HeLa Medium Control	44	44	44	44	44	44
Fungus Medium Control	0	0	0	0	0	0

*The enzyme activity is expressed in μmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

With respect to N-acetyl-β-glucosaminidase activity (Table 2), the Fungus flask medium remained essentially unchanged. Enzyme activity in the media of both of the controls was also constant. The medium with the HeLa plus Fungus combination was uniform in enzyme activity, except at the end of the experiment, where this activity dropped off completely. Increasing levels of N-acetyl-β-glucosaminidase with time were seen in the HeLa flask medium.

The Fungus medium and the Fungus Medium Control showed no activity in either of the galactosidase assays (Table 3). The HeLa medium and its control yielded essentially identical and consistent enzyme activities in the assay for β-D-galactosidase over the 60 hour period. The HeLa plus Fungus combination medium demonstrated a lack of

Table 2. Extracellular N-acetyl-β-glucosaminidase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	164	164	164	171	191	191
Fungus	42	42	42	44	44	44
HeLa plus Fungus	51	51	51	51	53	0
HeLa Medium Control	22	22	22	24	24	24
Fungus Medium Control	22	22	22	23.5	23.5	23.5

*The enzyme activity is expressed in μmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

Table 3. Extracellular β-D-galactosidase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	29	29	29	29	29	29
Fungus	0	0	0	0	0	0
HeLa plus Fungus	0	0	0	0	0	29
HeLa Medium Control	29	29	29	27.5	29	29
Fungus Medium Control	0	0	0	0	0	0

*The enzyme activity is expressed in μmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

β-D-galactosidase activity, except at the endpoint of the experiment, where enzyme activity first became measurable.

The Fungal Medium Control demonstrated consistency in phosphatase activities (Tables 4 and 5) with an absence of activity in the zero acid phosphatase tube. The Fungus medium yielded essentially consistent enzyme activities in all but the high reading in the zero acid phosphatase tube. Decreasing levels of alkaline and acid phosphatase were seen in the HeLa flask medium. The HeLa Medium Control flask showed a slight increase in acid phosphatase activity, with slight variability occurring in alkaline phosphatase. Phosphatase levels decreased in time in the medium with the HeLa plus Fungus.

Glutamic-Oxalacetic Transaminase, Glutamic-Pyruvic Transaminase

The extracellular activities of the glutamic transaminases in spent tissue culture medium are given in Tables 6 and 7. Standard curves represent decreasing levels of α-ketoglutaric acid, while either oxalacetic or pyruvic acid levels are increasing. Color intensity of the resulting hydrazones is proportional to transaminase activity. The Fungus and HeLa media controls exhibited identical and unchanging levels of GOT throughout the 60 hour testing period (Table 6). The media of the flasks with the Fungus and the HeLa plus Fungus also yielded consistent levels of GOT, with the combination flask medium

Table 4. Extracellular alkaline phosphatase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	28	23	23	18	18	14
Fungus	28	28	28	30	28	30
HeLa plus Fungus	83	32	32	32	30	9
HeLa Medium Control	98	102	106	102	102	100
Fungus Medium Control	21	21	21	23	21	21

*The enzyme activity is expressed in µmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

Table 5. Extracellular acid phosphatase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	112	94	91	72	72	72
Fungus	39	10	10	10	10	10
HeLa plus Fungus	39	28	10	10	10	10
HeLa Medium Control	6	8	8	10	10	10
Fungus Medium Control	0	10	10	10	10	10

*The enzyme activity is expressed in µmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

Table 6. Extracellular glutamic-oxalacetic transaminase activities in spent tissue culture medium*

HeLa	58	67	77	86	115	134
Fungus	48	48	48	48	48	48
HeLa plus Fungus	67	67	67	67	67	67
HeLa Medium Control	19	19	19	19	19	19
Fungus Medium Control	19	19	19	19	19	19

*The enzyme activity is expressed in μmol x 10,000/min, calculated from absorbance. Average of two assays.

having a somewhat higher level of enzyme activity. A noticeable increase in GOT was seen in the HeLa flask medium.

With respect to GPT levels (Table 7), all of the media in the flasks had constant levels of enzyme activity, with the highest level being obtained by the HeLa flask medium.

Alpha-Hydroxybutyric Dehydrogenase, Lactic Dehydrogenase

The extracellular activities of α-HBD in the various flasks are shown in Table 8. Both the LDH and the α-HBD standard curves illustrate enzyme activity as being inversely proportional to color intensity. Identical and constant levels of α-HBD were found in the media of both of the controls and in the medium with the HeLa plus Fungus.

Table 7. Extracellular glutamic-pyruvic transaminase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	211	211	211	211	211	211
Fungus	173	173	173	173	173	173
HeLa plus Fungus	154	154	154	154	154	154
HeLa Medium Control	19	19	19	19	19	19
Fungus Medium Control	154	154	154	154	154	154

*The enzyme activity is expressed in µmol x 10,000/ min, calculated from absorbance. Average of two assays.

Table 8. Extracellular α-hydroxybutyric dehydrogenase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	250	200	50	50	0	0
Fungus	350	350	350	250	250	250
HeLa plus Fungus	450	450	450	450	450	450
HeLa Medium Control	450	450	450	450	450	450
Fungus Medium Control	450	450	450	450	450	450

*The enzyme activity is expressed in µmol x 10,000/ min, calculated from absorbance. Average of two assays.

Decreasing levels were observed in the media of both the HeLa and the Fungus flasks.

The results of the LDH activities in the various flasks are not shown, because of the nature of the standard curve. The standard curve is constructed such that the amount of pyruvate is inversely proportional to the amount of LDH activity in the sample. The values obtained were greater than 0.960 µmol, which were well out of the range of sensitivity of the calibration curve recommended by Sigma (90).

Hydrogen Ion Concentrations and Cell Counts

In the medium with the HeLa plus Fungus (Table 9), the observed fungus counts ranged from 4.8×10^6 cells/ml at the zero hour to 7.5×10^6 cells/ml by the 60th hour. Viable HeLa cells remained attached to the plastic tissue culture flask and were not normally found floating in suspension. Dead fungi (1.5×10^5 cells/ml) were first noted at the 48th hour, as opposed to dead HeLa cells floating in suspension at the 24th hour. These dead HeLa cells were not intact by the 60th hour. The hydrogen ion concentration varied little during the first 48 hours, but by the 60th hour conditions inside the flask had become more alkaline.

The medium in the Fungus flask (Table 9) maintained a basic pH throughout the experiment, despite adjustment to pH 7.2-7.4 with HCl every 12 hours. The cell

Table 9. Cell counts and hydrogen ion concentrations in spent tissue culture medium

	Time (hrs)					
	0	12	24	36	48	60
HeLa						
Total cells/ml	0	0	0	1.5×10^5	1.5×10^5	1.5×10^5
Dead cells/ml	0	0	0	0	0	0
pH	7.2	7.4	7.2	7.0	7.0	7.1
Fungus						
Total cells/ml	4.4×10^6	5.1×10^6	5.3×10^6	6.4×10^6	7.5×10^6	7.5×10^6
Dead cells/ml	0	0	0	0	0	0
pH	8.4	8.4	8.4	8.4	8.4	8.4
HeLa/Fungus						
Total HeLa cells/ml	0	0	1.5×10^5	1.5×10^5	3.0×10^5	Lysis
Total Fungus cells/ml	4.8×10^6	5.5×10^6	5.6×10^6	6.7×10^6	7.5×10^6	7.5×10^6
Dead HeLa cells/ml	0	0	1.5×10^5	1.5×10^5	3.0×10^5	Lysis
Dead Fungus cells/ml	0	0	0	0	1.5×10^5	3.0×10^5
pH	7.6	7.6	7.7	7.6	7.6	8.4
HeLa MC						
pH	7.0	7.2	8.0	7.6	7.6	7.2
Fungus MC						
pH	7.0	7.2	8.0	7.6	7.6	7.2

concentrations ranged from 4.4 x 10^6 cells/ml to 7.5 x 10^6 cells/ml, and were 100% viable.

Hydrogen ion concentrations within the HeLa flask (Table 9) ranged from pH 7.0-7.4. Dead cells were not seen, but viable floating HeLa cells were seen by the 36th hour.

In both of the control flasks (Table 9), the hydrogen ion concentrations ranged from pH 7.0-8.0.

Experiment II - Fresh Medium

Alpha-D-glucosidase, β-D-glucosidase, N-acetyl-β-glucosaminidase, α-D-galactosidase, β-D-galactosidase, Alkaline Phosphatase, Acid Phosphatase

The extracellular activities of the above enzymes in fresh tissue culture medium with the exception of α-D-glucosidase and α-D-galactosidase are given in Tables 10 through 14.

A change in absorbance from zero to two hours yielded no measurable α-D-glucosidase or α-D-galactosidase activity in any of the flasks throughout the 60 hour testing period.

The HeLa flask medium and its control yielded identical enzyme activities in the assay for β-D-glucosidase (Table 10), excluding the final measurement. Decreasing levels of β-D-glucosidase activity were seen in all of the media in the flasks.

The HeLa medium showed increasing levels of N-acetyl-β-glucosaminidase activity (Table 11) with time. A slight increase was seen in the Fungus flask medium, and a decrease

Table 10. Extracellular β-D-glucosidase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	76	73	73	73	69	66
Fungus	76	73	73	73	69	60
HeLa plus Fungus	76	69	68	66	66	0
General Medium Control	76	73	73	73	69	58

*The enzyme activity is expressed in μmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

Table 11. Extracellular N-acetyl-β-glucosaminidase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	253	257	257	277	281	283
Fungus	260	263	263	263	263	265
HeLa plus Fungus	269	257	257	257	257	0
General Medium Control	240	240	242	242	242	244

*The enzyme activity is expressed in μmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

through the 48th hour in the HeLa plus Fungus flask medium. No appreciable change in enzyme activity occurred in the General Medium Control.

The media of both the HeLa flask and its control yielded essentially identical β-D-galactosidase activities (Table 12). The HeLa flask medium maintained a constant level of β-D-galactosidase activity, with a slightly

Table 12. Extracellular β-D-galactosidase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	44	39	40	40	40	40
Fungus	39	39	44	44	44	44
HeLa plus Fungus	40	40	40	42	44	11
General Medium Control	39	39	39	39	39	39

*The enzyme activity is expressed in μmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

increased level at the zero hour. The General Medium Control did not change from the zero hour to the 60th hour in its level of β-D-galactosidase. Increasing levels of activity were seen in the Fungus flask medium, and up to the 60th hour in the medium with the HeLa plus Fungus, where the activity dropped drastically.

Overall decreases in phosphatase activity (Tables 13 and 14) were observed in the media of the HeLa plus Fungus

Table 13. Extracellular alkaline phosphatase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	83	111	106	102	88	69
Fungus	98	120	106	116	102	83
HeLa plus Fungus	116	111	106	102	83	65
General Medium Control	153	130	106	102	88	69

*The enzyme activity is expressed in µmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

Table 14. Extracellular acid phosphatase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	39	54	61	54	72	58
Fungus	58	60	50	36	25	6
HeLa plus Fungus	76	61	47	47	36	10
General Medium Control	36	36	36	27	27	10

*The enzyme activity is expressed in µmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

flask and in the General Medium Control. In both the HeLa flask and the Fungus flask media, decreasing levels of alkaline phosphatase were seen after 12 hours. Variability with time was seen in acid phosphatase activity in the HeLa flask medium.

Glutamic-Oxalacetic Transaminase, Glutamic-Pyruvic Transaminase

The extracellular activities of the glutamic transaminases in fresh tissue culture medium are given in Tables 15 and 16. The HeLa, the Fungus, and the HeLa plus Fungus media exhibited increasing levels of GOT (Table 15) throughout the 60 hour period. The General Medium Control showed a constant level of enzyme activity.

Table 15. Extracellular glutamic-oxalacetic transaminase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	77	77	86	86	115	163
Fungus	67	67	86	96	96	96
HeLa plus Fungus	86	86	96	96	96	115
General Medium Control	67	67	67	67	67	67

*The enzyme activity is expressed in µmol x 10,000/min, calculated from absorbance. Average of two assays.

With respect to GPT levels (Table 16), the media for the Fungus, the HeLa plus Fungus, and the General Medium

Table 16. Extracellular glutamic-pyruvic transaminase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	77	96	96	96	111	111
Fungus	86	86	86	86	86	86
HeLa plus Fungus	86	86	86	86	86	86
General Medium Control	86	86	86	86	86	86

*The enzyme activity is expressed in µmol x 10,000/min, calculated from absorbance. Average of two assays.

control demonstrated identical and constant levels of GPT with time. Only the HeLa flask medium showed any kind of change in activity, which was an increase over the 60 hour period.

Alpha-Hydroxybutyric Dehydrogenase, Lactic Dehydrogenase

The extracellular activities of α-HBD in the various media are shown in Table 17. Increasing levels of activity were observed in both the HeLa and in the HeLa plus Fungus media. The General Medium Control demonstrated a constant level of activity, whereas the enzyme activity in the Fungus flask medium decreased with time.

The difficulty in measuring LDH in Experiment I also occurred in this experiment. The values obtained were well out of the range of the standard curve.

Table 17. Extracellular α-hydroxybutyric dehydrogenase activities in fresh tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	150	150	270	450	450	450
Fungus	250	150	150	150	150	10
HeLa plus Fungus	150	150	250	250	250	650
General Medium Control	150	150	150	150	150	150

*The enzyme activity is expressed in μmol x 10,000/min, calculated from absorbance. Average of two assays.

Hydrogen Ion Concentrations and Cell Counts

In the HeLa plus Fungus medium (Table 18), the observed fungus counts ranged from 1.1 x 10^6 cells/ml at the zero hour to 1.4 x 10^6 cells/ml by the 60th hour. Dead fungi and dead HeLa cells floating in suspension were both first noted at the 48th hour. Total lysis of HeLa cells occurred by the 60th hour. The hydrogen ion concentrations ranged from pH 7.0-8.4.

The Fungus medium (Table 18) remained at pH 7.8 for the final 48 hours. The cell concentrations ranged from 1.3 x 10^6 cells/ml to 1.6 x 10^6 cells/ml, and were 100% viable.

Hydrogen ion concentrations within the HeLa medium (Table 18) ranged from pH 7.0-7.4. Dead cells were not seen, but viable floating cells were seen by the 48th hour.

Table 18. Cell counts and hydrogen ion concentrations in fresh tissue culture medium

	Time (hrs)					
	0	12	24	36	48	60
HeLa						
Total cells/ml	0	0	0	0	1.5×10^5	1.5×10^5
Dead cells/ml	0	0	0	0	0	0
pH	7.4	7.3	7.2	7.2	7.0	7.0
Fungus						
Total cells/ml	1.3×10^6	1.3×10^6	1.0×10^6	1.1×10^6	1.0×10^6	1.6×10^6
Dead cells/ml	0	0	0	0	0	0
pH	8.0	7.8	7.8	7.8	7.8	7.8
HeLa/Fungus						
Total HeLa cells/ml	0	0	0	0	1.5×10^5	Lysis
Total Fungus cells/ml	1.1×10^6	1.1×10^6	1.0×10^6	1.1×10^6	1.0×10^6	1.4×10^6
Dead HeLa cells/ml	0	0	0	0	1.5×10^5	Lysis
Dead Fungus cells/ml	0	0	0	0	1.5×10^5	3.0×10^5
pH	8.0	7.6	7.2	7.0	7.0	8.4
General MC						
pH	7.6	7.6	7.6	7.6	7.6	7.6

In the General Medium Control (Table 18), the hydrogen ion concentration remained at pH 7.6.

Experiment III - Alkaline Phosphatase

Alkaline Phosphatase

The results of the enzyme activity after the addition of alkaline phosphatase into spent tissue culture medium are indicated in Table 19. Throughout the 60 hour testing period, results were identical and consistent in all but the medium in the HeLa flask. In the HeLa flask medium, a notable decrease in enzyme activity occurred with time.

Table 19. Extracellular alkaline phosphatase activities in spent tissue culture medium*

	Time (hrs)					
	0	12	24	36	48	60
HeLa	770	745	702	584	346	314
Fungus	752	752	752	752	752	752
HeLa plus Fungus	752	752	752	752	752	752
HeLa Medium Control	752	752	752	752	752	752
Fungus Medium Control	752	752	752	752	752	752

*The enzyme activity is expressed in µmol/2 hr, calculated from a change in absorbance over a 2 hour period. Average of two assays.

Hydrogen Ion Concentrations and Cell Counts

In the HeLa plus Fungus medium (Table 20), the fungus counts ranged from 5.25 x 10^6 cells/ml at the zero hour to 7.4 x 10^6 cells/ml by the 60th hour. Dead HeLa cells were first noted floating in suspension at the 24th hour, and dead fungi were first noted at the 48th hour. Lysis of HeLa cells occurred by the 60th hour. The hydrogen ion concentrations ranged from pH 7.2-8.5.

The medium in the Fungus flask (Table 20) remained at pH 8.0 until the 48th hour, when it dropped to pH 7.6. The cell concentrations ranged from 5.0 x 10^6 cells/ml to 7.3 x 10^6 cells/ml, and were 100% viable.

Hydrogen ion concentrations in the medium of the HeLa flask (Table 20) ranged from pH 6.9-7.1. Dead cells were not seen, but viable floating cells were seen by the 36th hour.

The hydrogen ion concentrations of the HeLa Medium Control ranged from pH 7.2-8.0, and in the Fungus Medium Control ranged from pH 7.3-8.0 (Table 20).

Experiment IV - Phagocytosis

After combining HeLa cells with *B. dermatitidis* cells, coverslips were examined at 1, 2, 3, 5, 9, 24, 51 and 77 hours to determine the total number of intracellular yeast bodies per 43X field. Rounded HeLa cells and yeasts were seen throughout the 77 hour period, usually in segregated clumps. By the 77th hour, poikilocytic HeLa cells were observed. The coverglass removed at the 5th hour contained

Table 20. Cell counts and hydrogen ion concentrations in spent tissue culture medium

	Time (hrs)					
	0	12	24	36	48	60
HeLa						
Total cells/ml	0	0	0	1.5×10^5	1.5×10^5	1.5×10^5
Dead cells/ml	0	0	0	0	0	0
pH	7.0	6.9	6.9	6.9	6.9	7.1
Fungus						
Total cells/ml	5.0×10^6	5.3×10^6	5.3×10^6	6.4×10^6	7.3×10^6	7.3×10^6
Dead cells/ml	0	0	0	0	0	0
pH	8.0	8.0	8.0	8.0	7.6	7.6
HeLa/Fungus						
Total HeLa cells/ml	0	0	1.5×10^5	1.5×10^5	3.0×10^5	Lysis
Total Fungus cells/ml	5.25×10^6	5.4×10^6	5.4×10^6	6.6×10^6	7.6×10^6	7.4×10^6
Dead HeLa cells/ml	0	0	1.5×10^5	1.5×10^5	3.0×10^5	Lysis
Dead Fungus cells/ml	0	0	0	0	1.5×10^5	3.0×10^5
pH	8.5	7.4	7.2	8.0	7.2	7.6
HeLa MC						
pH	8.0	7.2	7.4	7.4	7.4	7.4
Fungus MC						
pH	8.0	7.4	7.4	7.4	7.4	7.3

large, rounded, eosinophilic-staining HeLa cells, with 0-1 cells per 43X field which had phagocytized 1-2 yeast cells each. Phagocytized cells were not observed at any other time.

DISCUSSION

The interpretation of the experimental data will be discussed by comparing the activity in the media of the HeLa plus Fungus flask to the activity in the medium of each of the separate test flasks, and by comparing the activity in the medium of the separate test flasks to the activity of their respective media in the control flasks. Increased activity of any enzyme may indicate that the enzyme is no longer needed within the cells or that during the time of greatest activity within the cells and lowest extracellular levels, there was active synthesis of these enzymes, followed by release when they were no longer required intracellularly. A decrease in enzyme activity could result from the conservation of the enzymes by the cells which synthesize them in order that they be used within the cells.

Assays were performed to detect the presence of two glucosidases, but only β-D-glucosidase showed any activity. In Experiment I, the only test flask medium to demonstrate any activity was the HeLa cell medium. The HeLa Medium Control, when subtracted from its test flask medium, yielded slightly increasing levels of activity with time. The medium from the combination population flask, like the

Fungus flask medium, demonstrated no activity. Any enzyme activity present in the HeLa flask medium was quickly reduced. A factor involved in this reduction may have been the time element, since no enzyme activity was seen in the 11 day old control medium, as opposed to that which was seen in the 3 day old control medium. It is also possible that the medium in the HeLa flask may have been completely devoid of enzyme, since the 3 day old medium control demonstrated a level of enzyme production similar to that of the HeLa flask medium. The fetal calf serum may have been the only source of enzyme, which was degraded before the eleventh day.

The media in all flasks had β-D-glucosidase activity in fresh tissue culture medium, except medium in the combination flask, which was at the zero hour equal to the activity in the medium of each of the separate flasks, but then gradually decreased to an undetectable level of enzyme activity. This suggests destruction or inhibition of the enzyme activity by metabolites of one or both of the two cell types. Again, the actual production of exo-enzyme by one of the two cell types is questionable, as the Medium Control showed similar activity to the tests.

The overall significance here, and with the other enzymes assayed, lies not in the production of the enzyme but rather in its change in activity. At the zero hour in Experiment II, the combining of HeLa cells with fungal cells produced no change in β-D-glucosidase levels. At the 60th hour, enzyme activity was totally absent,

possibly destroyed or inhibited by the lytic HeLa cell products.

The highest level of N-acetyl-β-glucosaminidase in the first experiment was produced in the medium of the HeLa flask, which was approximately three times greater than that produced in the media by either the Fungus or the HeLa plus Fungus. A major portion of the enzyme produced by the HeLa cells was not preserved at any point in time up to 60 hours when the two cell types were combined. Whether all of the HeLa enzyme was destroyed and the fungus stimulated to produce slightly more enzyme in the presence of HeLa cells is speculative. The level of enzyme in the medium of the combination flask was lost at 60 hours, possibly because products of dead HeLa cells destroyed or inhibited the enzyme. The question of whether endogenous serum enzyme is the only source of enzyme is answered by the lower level of enzyme activity observed in the media controls as opposed to the media in the test flasks. The consistent and almost identical enzyme activity in both the 11 day and the 3 day controls might also lend support to the stability of the enzyme in the experimental system.

In the second experiment, the medium in the HeLa plus Fungus flask demonstrated variable enzyme activity for N-acetyl-β-glucosaminidase relative to the activity in the medium of the separate flasks. Yem and Wu (107) reported that this exoenzyme from bacteria does not vary significantly with respect to growth media, carbon source,

or phase of growth. If this is true, the variation in enzyme activity for the HeLa plus Fungus flask medium may be accounted for by metabolic products from the cell types themselves. The HeLa plus Fungus flask medium had the greatest enzyme activity of the three test flasks' media at the zero hour, probably due to the combining of the two cell types. For the next 24 hours, all three test media were at about equal levels of activity, indicating that some of the enzyme present in the HeLa plus Fungus flask medium at the zero hour was inhibited or destroyed by the 12th hour. At 60 hours, HeLa cell death occurred. Lysis of these cells at this time was accompanied by a total loss of the enzyme activity in the combination flask medium, probably due to the HeLa lytic products. The Medium Control demonstrated that endogenous enzyme was already present in the fetal calf serum in fresh tissue culture medium. The first experiment demonstrated a significant decrease in enzyme activity upon incubation at 37 C for at least 3 days. The controls were stable for each experimental condition.

Assays were performed to detect the presence of two galactosidases, but only β-D-galactosidase activity in the HeLa flask medium was inhibited or destroyed when the fungus was added to the HeLa cells. The enzyme itself may have been present in the fetal calf serum, since the enzyme was present in the HeLa Medium Control flask. Apparently, a loss of enzyme activity occurred at some time before 11 days of incubation at 37 C. At 60 hours,

when HeLa cell death occurred, intracellular products of lysis or removal of inhibitor may have accounted for the sudden appearance of this enzyme in the HeLa plus Fungus medium.

In fresh tissue culture medium, the β-D-galactosidase activities in the media of the respective flasks showed only slight variations. The values for this enzyme in the medium of the HeLa plus Fungus flask were similar to those observed both in the media of the separate test flasks and in the control through 48 hours. Enzyme activity in the medium of the combination flask was partially inhibited or destroyed in 60 hours at the time of HeLa cell lysis, probably because of intracellular HeLa cell metabolites causing a change. The presence of enzyme in the control again suggests exogenous β-D-galactosidase residing in the serum of both experiments.

Intracellular β-galactosidase activity has been reported to be affected by the ionic composition of the medium (4). Salts containing $SO_4^{=}$, Cl^{-}, Na^{+} and Mg^{++} stimulate enzyme activity. Intracellular β-galactosidase activity in yeasts has also been reported to increase near the end of the cell cycle (106). Beta-galactosidase is an inducible enzyme. The experimental design does not clarify the questionable presence of the enzyme. In the absence of lactose in the medium as the major carbon source, β-galactosidase is expected to be present at very low levels inside the cells, and not to be secreted to the external environment. No significant increase in

extracellular activity was seen at any time during the experiments.

Neither one of the organisms together or separately showed any extracellular activity toward PNP derivatives of α-D-glucoside or α-D-galactoside. These enzymes may be bound to the cell and do not escape into the medium without cell disintegration. It is also possible that cell bound enzymes are located outside an impermeable cytoplasmic membrane. Only certain enzymes, called "partially cell-bound enzymes" by Pollock (66), may diffuse through pores of the cell wall.

According to Mitchell and Moyle (57), a small amount of potentially extracellular enzyme is expected to be located on the outer layers of the cell. If this is the case, then enzymatic secretion would be dependent upon the cell wall. Enzyme release from the cell would be partially or completely prevented. Mitchell and Moyle concluded that the amount of enzyme produced depends on the properties both of the enzyme and of the cell wall.

The results of the alkaline phosphatase assays of the HeLa plus Fungus activity were greater than in the separate flasks through 48 hours, particularly at the zero hour. This suggests an additive increase in activity due to combining the two cell types. The increased activity of the enzyme from the medium in the combination flask dropped to the level of activity in the media of the separate flasks after the zero hour, indicating enzyme inhibition or degradation. Intracellular products of

lysed HeLa cells may have accounted for a sudden disappearance of the activity of the combination flask at 60 hours. Activity in the medium of the Fungus flask and its control were relatively consistent, whereas the enzyme in the medium of the HeLa flask appeared to be decreasing with time, despite the presence of exogenous alkaline phosphatase. The appearance of extracellular alkaline phosphatase is associated with the nutritional level of the organism with respect to phosphorus. As long as orthophosphate is available, no enzyme is released into the surrounding medium.

In the second experiment, the alkaline phosphatase activity in the medium of the combination HeLa plus Fungus flask was again greater than the media in either of the separate flasks at the zero hour. Later on, a decrease in enzyme activity caused the level in the medium of the HeLa plus Fungus flask to approximate that within the HeLa flask. Intracellular products of lysed HeLa cells may have been responsible for some destruction or inhibition of enzyme in the HeLa plus Fungus medium at 60 hours.

Another interpretation is suggested by looking at the General Medium Control. The presence of exogenous alkaline phosphatase may have been due to inhibition or degradation by metabolic products of either organism. This seems unlikely as the subtracting of the levels of activities in the media of the controls from both the HeLa and the Fungus flasks demonstrate increasing activities or leveling off with time.

A third experiment was done to observe the effects on a specific amount of alkaline phosphatase solution placed into the spent medium system. As in Experiment I, the enzyme in the medium of the HeLa flask was degraded. All other flasks were consistent and identical, thus demonstrating no increased activity. The degradation or inhibition of pure alkaline phosphatase in the HeLa flask in this third experiment also ruled out the occurrence of artifactual decrease of enzyme in the first experiment.

The level of acid phosphatase in the first experiment was found to decrease with time in both media of the HeLa and the Fungus flasks, despite a slight increase in the controls. The relatively high levels of enzyme present initially in the tests seem to indicate removal of inhibitor or production of enzyme by the organism as opposed to only exogenous enzyme being present. Combining the two cell types caused a decrease in enzyme activity which continued through 60 hours. Acid phosphatase was not detected by 60 hours in the medium of the HeLa plus Fungus flask.

In fresh tissue culture medium, the HeLa flask medium, after the control values were subtracted, showed an increase in acid phosphatase activity with time. This enzyme in fungi is either strictly or partially repressed (34,62,71, 88,103). In other words, acid phosphatase is produced in small quantities in the presence of phosphate, but markedly in its absence. Nonspecific acid phosphatase was reported to be localized extracellularly by Linko and Schmidt (96)

and to be derepressed by lowering the concentration of inorganic phosphate in the medium. Combining the two cell types, HeLa and fungus, yielded variable results for acid phosphatase. Only at the zero hour was the HeLa plus Fungus enzyme activity greater than that in the medium of the separate flasks. Following this point in time, enzyme produced by the fungus and/or the HeLa cells was degraded or inhibited, possibly by cellular metabolites, when the cell types were combined. This decrease in activity was expected, as the enzyme in the medium of the Fungus flask was gradually being destroyed.

In the first experiment, glutamic-oxalacetic transaminase (GOT) stability with time and incubation at 37 C was observed, except in the medium of the HeLa flask, which showed increasing activity. The HeLa plus Fungus medium exhibited greater GOT activity than either the Fungus or the HeLa medium alone, but after 12 hours the combination was less than the HeLa medium. This seems to indicate an immediate partial loss of activity in the HeLa plus Fungus medium, possibly increasing with time. The increased secretion of enzyme by the HeLa cells may account for the consistent level in the medium of the combination flask, such that a decrease in activity was masked by HeLa cell enzyme production. Again, in this experimental system, it is impossible to identify which cell type is producing and which is destroying or inhibiting any enzyme. Electrophoretic studies would assist in answering such questions.

The second experiment revealed stability of the GOT enzyme with respect to the control. In this case, both the Fungus medium and especially the HeLa medium showed increasing levels of enzyme activity with respect to the control medium. The combination medium was greater in activity than either of the separate media flasks through 48 hours, possibly due to the additive effect of the two cell types or the stimulation of one cell type by another to produce enzyme. Natural inhibitors of the enzyme, namely L-aspartate, L-glutamate and succinate, were not known to be present in this particular tissue culture medium.

Glutamic-pyruvate transaminase (GPT) activity in the first experiment was produced in large amounts by the HeLa cells and lesser by the fungal cells. Although consistent levels of enzyme were seen in the media controls, the level appeared to be greater in incubated material. Unlike many of the other enzymes, in spent material both of the separate media flasks demonstrated higher levels of enzyme activity than the combined flask medium. This loss of activity in the HeLa plus Fungus flask medium seems to have been due to the cells or to their metabolic products, as enzyme inhibitors such as L-glutamate, L-alanine, and L-aspartate were not present in MEM. Although a loss of enzyme was observed upon combining the two cell types, no further decrease was seen over the 60 hour period.

Glutamic-pyruvate transaminase activity in the second experiment varied with time only in the medium of the HeLa

flask, where an increase was seen. Apparently, combining HeLa cells with fungal cells in fresh tissue culture medium produced an enzyme level equal to that produced by the fungal cells alone. This activity stayed constant and equal to that of the control. The combination of the two cell types seemed to effect a degradation or inhibition of enzyme. Whether or not the HeLa cells produced an enzyme that was being degraded cannot be certain without electrophoretic studies. The combined level of activity, although equal to that of the medium of the Fungus flask, was also equal to the control medium activity. The results indicate enzyme production by only HeLa cells or that an enzyme inhibitor was present in the other flask.

Alpha-hydroxybutyric dehydrogenase (α-HBD) activity was stable with respect to the controls. An overall change in enzyme activity came about as a decrease, particularly in the medium of the HeLa flask. Despite the occurrence of enzyme inhibition or degradation in media of both the HeLa and Fungus flasks, the medium of the HeLa plus Fungus activity remained quite high and consistent. These results suggest at least two possibilities. The two cell types together may have produced an increased level of α-HBD. A more plausible explanation is suggested by observing the results of the controls. Similar activities in the HeLa plus Fungus medium and in the media controls suggest only a lack of enzyme decrease. The immediate degradation or inhibition of enzyme occurring

in media of the separate flasks is not seen in the HeLa plus Fungus medium.

Results of the second experiment also indicate α-HBD stability with respect to the control, but considerably lower levels of activity were observed than those in spent MEM. In this experiment, the HeLa flask medium increased in enzyme activity, while the Fungus flask medium decreased. A rapid lowering of α-HBD was observed at 60 hours in the Fungus flask medium. In the combination flask at 60 hours, it is possible that the HeLa cells may have been contributing a great amount of detectable enzyme, but the source was probably intracellular, due to lysis.

The number of variables that can affect enzyme levels is virtually endless. In cells that are in culture, these include nutritional, developmental, stage of the cell cycle, and viral infection (85). Whether an observed difference in measurable activity results from a difference in catalytic activity of pre-existing enzyme or an altered content of enzyme can be elucidated by studying the mechanism for an altered enzyme level. Once it is established that the difference in enzyme activity results from a difference in the content of enzyme protein, the next logical question is whether the increase or decrease is due to an alteration in the rate of synthesis, the rate of degradation, or both. Inhibitors from yeasts and/or HeLa cells probably influence the activities of these enzymes. Some of these questions could be answered

by the incorporation of isotope into specific protein and combining these results with electrophoretic studies. This is necessary for developing a better understanding of the extracellular enzyme activities of the host cells and of the pathogenic fungal cell.

LITERATURE CITED

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1. Almin, K. E., K.-E. Eriksson, and B. Pettersson. 1975. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum (Chrysosporium lignorum)* for the breakdown of cellulose. 2. Activities of the five endo-1,4-β-glucanases towards carboxymethylcellulose. Eur. J. Biochem. 51: 207-211.

2. Armentrout, V. N., G. Hänssler, and D. P. Maxwell. 1976. Acid phosphatase localization in the fungus *Whetzelinia sclerotiorum*. Arch. Microbiol. 107: 7-14.

3. Arnaud, N., D. A. Bush, and M. Horisberger. 1976. Study of an intracellular α-galactosidase from the thermophilic fungus *Penicillium duponti*. Biotech. and Bioeng. 18: 581-585.

4. Baccino, F. M., M. F. Zuretti, and L. Pernigotti. 1975. Stimulation of rat liver β-galactosidase activity by ions. Biochem. J. 151: 567-573.

5. Baker, R. D. 1942. Experimental blastomycosis in mice. Am. J. Pathol. 16: 463-477.

6. Bakerspigel, A. 1953. Soils as a storage medium for fungi. Mycologia 45: 596-604.

7. Bauer, H., and E. Sigarlakie. 1973. Cytochemistry on ultrathin frozen sections of yeast cells. J. Microscopy 99: 205-218.

8. Beneke, E. S., and A. L. Rogers. 1970. *Medical Mycology Manual*, 3rd Ed., page 130, Burgess Publishing Co., Minneapolis, Minn.

9. Beneke, E. S., R. W. Wilson, and A. L. Rogers. 1969. Extracellular enzymes of *Blastomyces dermatitidis*. Mycopathologia 39: 325-328.

10. Bergstrom, V. M., Nugent, Grace, and M. C. Snider. 1937. Blastomycosis. Report of a case with involvement of the skin and bones. Arch. Dermat. and Syph. 36: 70-76.

11. Bowen, J. T., and S. B. Wolbach. 1906. A case of blastomycosis: The results of culture and inoculation experiments. J. Med. Research 15: 167-177.

12. Bulmer, G. S., and Y. T. Li. 1966. Enzymatic activities in *Calvatia cyathiformis* during and after meiosis. Mycologia 58: 555-561.

13. Burt, W. R., and J. Cazin, Jr. 1976. Production of extracellular ribonuclease by yeasts and yeastlike fungi, and its repression by orthophosphate in species of *Cryptococcus* and *Tremella*. J. Bacteriol. 125: 955-960.

14. Cox, R. A., and G. K. Best. 1972. Cell wall composition of two strains of *Blastomyces dermatitidis* exhibiting differences in virulence for mice. Infect. Immun. 5: 449-453.

15. Cox, R. A., L. R. Mills, G. K. Best, and J. F. Denton. 1974. Histologic reactions to cell walls of an avirulent and a virulent strain of *Blastomyces dermatitidis*. J. Infect. Dis. 129: 179-186.

16. Davis, B. F. 1911. The immunological reactions of oidiomycosis (blastomycosis) in the guinea-pig. J. Infect. Dis. 8: 190-240.

17. Davis, T. E., and J. E. Domer. 1976. Glucanase production by *Histoplasma capsulatum* and *Blastomyces dermatitidis*. Abstracts of the Annual Meeting, American Society for Microbiology, Atlantic City, N.J.

18. DeMonbreun, W. A. 1935. Experimental chronic cutaneous blastomycosis in monkeys. Arch. Derm. and Syph. 31: 831-854.

19. Denton, J. F., and A. F. DiSalvo. 1964. Isolation of *Blastomyces dermatitidis* from natural sites at Augusta, Georgia. Am. J. Trop. Med. Hyg. 13: 716-722.

20. Denton, J. F., E. S. McDonough, L. Ajello, and R. J. Ausherman. 1961. Isolation of *Blastomyces dermatitidis* from the soil. Science 133: 1126-1127.

21. Dimond, R. L., and W. F. Loomis. 1976. Structure and function of β-glucosidases in *Dictyostelium discoideum*. J. Biol. Chem. 251: 2680-2687.

22. DiSalvo, A. F., and J. F. Denton. 1963. Lipid content of four strains of *Blastomyces dermatitidis* of different mouse virulence. J. Bacteriol. 85: 927-931.

23. Duque, H. O. 1946-47. Cultivo de tejidos y producción de granulomas experimentales micósicos sobre fibroblastos cultivados in vitro. Bol. Clin. Fac. de Med. Univ. Antiqua (Colombia) 9: 392-395.

24. Ebert, J. W., and V. Jones. 1971. Experimental canine histoplasmosis and blastomycosis. Mycopathologia 45: 285-300.

25. Eberhart, B., D. F. Cross, and L. R. Chase. 1964. β-glucosidase system of *Neurospora crassa*. I. β-glucosidase and wild-type strains. J. Bacteriol. 87: 761-770.

26. Ericksson, K.-E., and B. Pettersson. 1975. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum (Chrysosporium lignorum)* for the breakdown of cellulose. 3. Purification and physico-chemical characterization of an exo-1,4-β-glucanase. Eur. J. Biochem. 51: 213-218.

27. Fennell, D. I., K. B. Raper and M. H. Flickinger. 1950. Further investigations on the preservation of mold cultures. Mycologia 42: 135-147.

28. Fleet, G. H., and H. J. Phaff. 1975. Glucanases in *Schizosaccharomyces*. Isolation and properties of an exo-β-glucanase from the cell extracts and culture fluid of *Schizosaccharomyces japonicus* var. *versatilis*. Biochim. Biophys. Acta 401: 318-332.

29. Friebe, B., and A. W. Holldorf. 1975. The control of extracellular 1,3-β-glucanase activity in the Basidiomycete species QM806. Biochemical Society Transactions. Edinburgh. 3: 994-996.

30. Friedheim, E. A. H., and B. Baroni. 1929. L'immunité et la receptivité naturelles vis-à-vis de 1' Actinomyces asteroides etudiées en culture de tissu. Compt. rend. soc. biol. 100: 1089-1091.

31. Hamburger, W. W. 1907. A comparative study of four strains of organisms isolated from four cases of generalized blastomycosis. J. Infect. Dis. 4: 201-209.

32. Heinrichová, K., and L. Rexová-Benková. 1976. Purification and characterization of an extracellular exo-D-galacturononase of *Aspergillus niger*. Biochim. Biophys. Acta 422: 349-356.

33. Hempel, H., and N. L. Goodman. 1975. Rapid conversion of *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Sporothrix schenckii* in tissue culture. J. Clin. Microbiol. 1: 420-424.

34. Heredia, C. F., F. Yen and A. Sols. 1963. Role and formation of the acid phosphatase in yeast. Biochem. Biophys. Res. Commun. 10: 14-18.

35. Hill, G. A., and S. Marcus. 1957. Nature of resistance in mouse histoplasmosis. Bacteriol. Proc. 81.

36. Hinton, A., and S. L. Silberg. 1957. Use of HeLa tissue culture medium in the laboratory identification of pathogenic fungi. Am. J. Clin. Pathol. 28: 618-621.

37. Hislop, E. C., V. M. Barnaby, C. Shellis, and F. Laborda. 1974. Localization of α-L-arabinofuranosidase and acid phosphatase in mycelium of *Sclerotinia fructigena*. J. Gen. Microbiol. 81: 79-99.

38. Hopsu-Havu, V. K., C. E. Sonck, and E. Tunnela. 1972. Production of elastase by pathogenic and non-pathogenic fungi. Mykosen 15: 105-110.

39. Howard, D. H. 1964. Intracellular behavior of *Histoplasma capsulatum*. J. Bacteriol. 87: 33-38.

40. Howard, D. H. 1965. Intracellular growth of *Histoplasma capsulatum*. J. Bacteriol. 89: 518-523.

41. Howard, D. H. 1967. Effect of temperature on the intracellular growth of *Histoplasma capsulatum*. J. Bacteriol. 93: 438-444.

42. Howard, D. H., and R. L. Herndon. 1960. Tissue cultures of mouse peritoneal exudates inoculated with *Blastomyces dermatitidis*. J. Bacteriol. 80: 522-527.

43. Huang, K., and M. E. Landay. 1969. Enhancement of the lethal effects of endotoxins by interferon inducers. J. Bacteriol. 100: 1110-1111.

44. Iten, W., and Matile. 1970. Role of chitinase and other lysosomal enzymes of *Coprinus lagopus* in the autolysis of fruiting bodies. J. Gen. Microbiol. 61: 301-309.

45. Kiesel, A. 1911. Z. physiol. Chem. 75: 169.

46. Kim, Y. P., K. Adachi, and D. Chow. 1962. Leucine aminopeptidase in *Candida albicans*. J. Invest. Derm. 38: 115-116.

47. Komano, T. 1975. Formation of multiple forms of acid and alkaline phosphatases in relation to the culture age of *Aspergillus niger*. Plant and Cell Physiol. 16: 643-658.

48. Kurup, P. V. 1963. Histochemical demonstration of alkaline phosphatase in *Candida*. Current Science 32: 408-410.

49. Larsh, H. W., A. Hinton, and S. L. Silberg. 1956. Conversion and maintenance of *Histoplasma capsulatum* in tissue culture. Proc. Soc. Exptl. Biol. Med. 93: 612-615.

50. Larsh, H. W., A. Hinton, and S. L. Silberg. 1957-1958. The use of the tissue culture method in evaluating antifungal agents against systemic fungi. In *Antibiotics Annual*, pp. 988-991, Medical Encyclopedia, Inc., New York.

51. Larsh, H. W., and C. C. Shepard. 1958. HeLa cells and *Histoplasma capsulatum*. Phagocytosis and subsequent intracellular growth. J. Bacteriol. 76: 557-563.

52. Levine, S., and Z. J. Ordal. 1946. Factors influencing the morphology of *Blastomyces dermatitidis*. J. Bacteriol. 52: 687-694.

53. Lubarsky, R., and O. A. Plunkett. 1955. In vitro production of the spherule phase of *Coccidioides immitis*. J. Bacteriol. 70: 182-186.

54. Merchant, D. J., R. H. Kahn, and W. H. Murphy. 1965. *Handbook of Cell and Organ Culture*. Burgess Publishing Co., Minneapolis, Minn. Second Printing.

55. Michalski, C. J., and E. S. Beneke. 1969. Enzymatic activities during basidiocarp development of *Pleurotus ostreatus*. Mycologia 61: 1041-1047.

56. Michelson, I. D. 1928. Blastomycosis. A pathologic and bacteriologic study. J. Am. Med. Assoc. 91: 1871-1876.

57. Mitchell, P., and J. Moyle. 1959. Permeability of the envelopes of *Staphylococcus aureus* to some salts, amino acid and non-electrolyte. J. Gen. Microbiol. 20: 434-441.

58. Montes, L. F., and W. H. Wilborn. 1970. Acid phosphatase activity during phases of growth in *Candida albicans*. Internat. J. of Dermatology 9: 220-225.

59. Notario, V., T. G. Villa, T. Benítez, and J. R. Villanueva. 1976. β-Glucanases in the yeast *Cryptococcus albidus* var. *aerius*. Production and separation of β-glucanases in asynchronous cultures. Can. J. Microbiol. 22: 261-268.

60. Notario, V., T. G. Villa, and J. R. Villanueva. 1976. β-Xylosidases in the yeast *Cryptococcus albidus* var. *aerius*. Can. J. Microbiol. 22: 312-315.

61. Odds, F. C., and A. Trujillo-Gonzales. 1974. Acid phosphatase levels in the genus *Candida* and their application to the taxonomy and identification of pathogenic *Candida* species. Sabouraudia 12: 287-294.

62. Ohta, Y., K. Ikeda, and S. Ueda. 1968. Production of phosphatase by *Aspergillus awamori* var. *kawachii* in a low phosphate medium. Appl. Microbiol. 16: 973-980.

63. O'Sullivan, J., and G. E. Mathison. 1971. The localization and secretion of a proteolytic enzyme complex by the dermatophyte fungus *Microsporum canis*. J. Gen. Microbiol. 68: 319-326.

64. Peck, R. L. 1947. The lipids of fungi with special reference to pathogenic fungi, p. 167-188. In W. J. Nickerson (ed.), *Biology of Pathogenic Fungi*. Chromica Botanica Co., Waltham, Mass.

65. Peck, R. L., and C. R. Hauser. 1938. Chemical studies of certain pathogenic fungi. I. The lipids of *Blastomyces dermatitidis*. J. Amer. Chem. Soc. 60: 2599-2603.

66. Pollock, M. R. 1962. *Exoenzymes. The Bacteria: A Treatise on Structure and Function*. Vol. IV. *The Physiology of Growth*. Academic Press, New York.

67. Purnell, D. M., and G. M. Martin. 1971. *Aspergillus nidulans*: Association of certain alkaline phosphatase mutations with decreased virulence for mice. J. Infect. Dis. 123: 305-306.

68. Randall, C. C., and A. L. Hackney. 1953. Observations on human tissue cultures naturally infected by *Histoplasma capsulatum*. Am. J. Pathol. 29: 861-869.

69. Randall, C. C., and D. J. Turner. 1953. Cultivation of yeast in Earl's "L" strain mouse cells in vitro. Proc. Soc. Exptl. Biol. Med. 83: 584-585.

70. Raudaskoski, M. 1975. Acid phosphatase activity in the wild-type and B-mutant hyphae of *Schizophyllum commune*. J. Gen. Microbiol. 94: 373-379.

71. Rautanen, N., and V. Kärkkäinen. 1951. On the phosphatase activity of low-phosphorous *Torulopsis utilis*. Acta Chem. Scand. 5: 1216-1217.

72. Remold, H., H. Fasold, and F. Staib. 1968. Purification and characterization of a proteolytic enzyme from *Candida albicans*. Biochim. Biophys. Acta 167: 399-406.

73. Ricketts, H. T. 1901. Oidiomycosis (blastomycosis) of the skin and its fungi. J. Med. Research 6: 373-546.

74. Rippon, J.W. 1968. Extracellular collagenase from *Trichophyton schoenleinii*. J. Bacteriol. 95: 43-46.

75. Rippon, J.W. 1971. Differences between "+" and "-" strains of keratinophilic fungi. Recent Advances in Microbiology 7: 473-475.

76. Rippon, J. W. 1974. *Medical Mycology: The Pathogenic Fungi and the Pathogenic Actinomycetes*, page 298, W. B. Saunders Co., Philadelphia, Pa.

77. Rippon, J. W., and E. D. Garber. 1969. Dermatophyte pathogenicity as a function of mating type and associated enzymes. J. Invest. Derm. 53: 445-448.

78. Rippon, J. W., and D. P. Varadi. 1968. The elastase of pathogenic fungi and actinomycetes. J. Invest. Derm. 50: 54.

79. Rosenthal, S. A. 1964. Enzymatic studies with pathogenic fungi. Newsletter of the Medical Mycology Society of New York, Vol. 3, No. 1, pages 1-3, September.

80. Roy, I., and J. W. Landau. 1971. Polymorphism of malate dehydrogenase in cytoplasmic and mitochondrial fractions of *Blastomyces dermatitidis*. Sabouraudia 9: 39-42.

81. Rudick, M. J., and A. D. Elbein. 1975. Glycoprotein enzymes secreted by *Aspergillus fumigatus*: Purification and properties of a second β-glucosidase. J. Bacteriol. 124: 534-541.

82. Salfelder, K. 1965. Experimental cutaneous North American blastomycosis in hamster. J. Invest. Dermatol. 45: 409-418.

83. Salvin, S. B. 1949. Phase-determining factor in *Blastomyces dermatitidis*. Mycologia 41: 311-319.

84. Salvin, S. B. 1952. Endotoxin in pathogenic fungi. J. Immunology 69: 89-99.

85. Schimke, R. T., in *Axenic Mammalian Cell Reactions*, G. L. Tritsch, ed., Dekker, New York, 1969, p. 181.

86. Schmidt, G., G. Bartsch, M.-C. Laumont, T. Herman, and M. Liss. 1963. Acid phosphatase of baker's yeast: An enzyme of the external cell surface. Biochemistry 2: 126-131.

87. Shibata, K. 1903-1904. Beitr. chem. Physiol. Path. 5: 384.

88. Shieh, T. R., R. J. Wodzinski, and J. H. Ware. 1969. Regulation of the formation of acid phosphatases by inorganic phosphate in *Aspergillus ficuum*. J. Bacteriol. 100: 1161-1165.

89. Sigma Chemical Company. 1974. Technical Bulletin No. 495. P.O. Box 14508, St. Louis, Mo.

90. Sigma Chemical Company. 1975. Technical Bulletin No. 500. P.O. Box 14508, St. Louis, Mo.

91. Sigma Chemical Company. 1975. Technical Bulletin No. 505. P.O. Box 14508, St. Louis, Mo.

92. Staib, F. 1965. Serum proteins as nitrogen source for yeastlike fungi. Sabouraudia 4: 187-193.

93. Stanley, V. C., and R. Hurley. 1967. Growth of *Candida* species in cultures of mouse epithelial cells. J. Path. Bact. 94: 301-315.

94. Streamer, M., K.-E. Eriksson, and B. Pettersson. 1975. Extracellular enzyme system utilized by the fungus *Sporotrichum pulverulentum (Cryso-sporium lignorum)* for the breakdown of cellulose. Functional characterization of five endo-1,4-β-glucanases and one exo-1,4-β-glucanase. Eur. J. Biochem. 59: 607-613.

95. Sumner, J. B., and L. O. Poland. 1933. Proc. Soc. Exptl. Biol. Med. 30: 553.

96. Suomalainen, H., M. Linko, and E. Oura. 1960. Changes in the phosphatase activity of baker's yeast during the growth phase and location of phosphatases in the yeast cell. Biochim. Biophys. Acta (Amst.) 37: 482-490.

97. Taylor, J. J. 1962. Demonstration and possible significance of urease in human pathogenic fungi. Nature 194: 403-404.

98. Taylor, J. J. 1964. Enhanced toxicity in chemically altered *Blastomyces dermatitidis*. J. Bacteriol. 87: 748-749.

99. Taylor, J. J., and C. E. Johnson. 1962. Urease activities in cultures and extracts of *Blastomyces dermatitidis*. Enzymologia 25: 87-93.

100. Ueda, Y., and Y. Oshima. 1974. A constitutive mutation, phoT, of the repressible acid phosphatase synthesis with inability to transport inorganic phosphate in *Saccharomyces cerevisiae*. Molec. gen. Genet. 136: 255-259.

101. Wagoner, N. E., A. L. Morehart, and H. W. Larsh. 1965. Improved techniques for the reversion of *Histoplasma capsulatum* in tissue culture. Mycopathologia 26: 117-122.

102. Wang, C. J. K., and J. Schwarz. 1959. Phagocytosis of yeast cells in vitro. Am. J. Pathol. 35: 901-907.

103. Weimberg, R., and W. L. Orton. 1963. Repressible acid phosphomonoesterase and constitutive pyrophosphatase of *Saccharomyces mellis*. J. Bacteriol. 86: 805-813.

104. Wilson, R. W., and D. J. Niederpruem. 1967. Control of β-glucosidases in *Schizophyllum commune*. Can. J. Microbiol. 13: 1009-1020.

105. Wilson, C. L., D. L. Stiers, and G. G. Smith. 1970. Fungal lysosomes or spherosomes. Phytopathology 60: 216-227.

106. Yashphe, J., and H. O. Halvorson. 1976. β-D-Galactosidase activity in single yeast cells during cell cycle of *Saccharomyces lactis*. Science 191: 1283-1284.

107. Yem, D. W., and H. C. Wu. 1976. Purification and properties of β-N-acetylglucosaminidase from *Escherichia coli*. J. Bacteriol. 125: 324-331.

108. Zaikina, N. A., and N. P. Elinov. 1968. Fungal plasmacoagulase. Mycopathologia 35: 10-16.