COMPARATIVE STUDY OF PROTEIN FRACTIONS AND ENZYMES IN THE CULTURE FILTRATES OF HISTOPLASMA CAPSULATUM DARLING AND HISTOPLASMA DUBOISII VANBREUSEGHEM

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Parimondh Khanjanasthiti

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Everett S. Beneke

Major professor
Everett S. Beneke

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ABSTRACT

COMPARATIVE STUDY OF PROTEIN FRACTIONS AND ENZYMES IN THE CULTURE FILTRATES OF HISTOPLASMA CAPSULATUM DARLING AND HISTOPLASMA DUBOISII VANBREUSEGHEM

By

Parimondh Khanjanasthiti

The purpose of this research was to study the taxonomic relationship between Histoplasma capsulatum Darling, 1906, and H. duboisii Vanbreuseghem 1952, based on an investigation of the protein fractions and certain enzymes in the culture filtrates. This information may be useful in a more definitive classification of the etiological agent of the large-form of histoplasmosis, H. duboisii which has been considered by some as a stable variety of H. capsulatum.

Disc electrophoresis was performed on soluble proteins extracted from culture filtrates after 10, 20, and 30 days at 25 C and 37 C from H. capsulatum type "A" and "B" and H. duboisii. More fractions were produced in the older culture filtrates. After 30 days at 25 C, H. capsulatum type A had 3 fractions; type B, 7; and H. duboisii had 5 fractions. The fractions of type A were common with 3 in B, while 3 of the fractions

of H. duboisii were common with 3 in B, but different from A. At 37 C, type A had only 5 fractions, type B had only 2, both of which were homologous with type A, while H. duboisii produced 3 fractions, 1 of which was common for type A and B.

Structural membrane proteins in the cell extract of the two species grown in CYPG medium at 37 C produced patterns very similar for 9 strains of H. capsulatum and 5 strains of H. duboisii. Histoplasma capsulatum type A and type B had almost identical patterns with a range of 8 to 10 homologous bands.

Seven paranitrophenol substrates were used to assay the culture filtrates and agar media for extracellular enzyme activities from H. capsulatum and H. duboisii. The mycelial phase of both species produced high enzyme activities in PNP-alpha- and PNP-beta-D-glucoside substrates. Histoplasma capsulatum type A had 2 to 8 times greater activity in BHI agar than type B for alpha- and beta-PNPGase. In the yeast phase only, H. duboisii had high activity for these enzymes. The kinetics, inhibitors, optimal temperature and pH were studied for alpha- and beta-PNP-glucosidases produced by H. duboisii.

Histoplasma duboisii produced high PNP-alkaline phosphatase activity in CPYG agar at 37C while it was low for H. capsulatum.

The study of fine structures of young hyphae from H. duboisii showed similar organelles to those in H. capsulatum. Woronin bodies were found near the septum in the hyphae of H. capsulatum but were not found in the sections made of hyphae of H. duboisii.

Phenol red in a modified CPYG medium differentiated most strains of H. capsulatum from H. duboisii by a more rapid change in the pH, resulting in a color change in the medium from yellow to red.

The similarities in enzyme studies and electrophoretic protein patterns of a number of strains of the two species appears to support the proposal that the large yeast cell form of histoplasmosis should be considered H. capsulatum Darling, 1906 var. duboisii (Vanbreuseghem, 1952) Ciferri 1960.

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By

Parimondh Khanjanasthiti

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To my wife Monta
and my son Ton

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INTRODUCTION

The relationship of the two species, Histoplasma capsulatum Darling 1906 and H. duboisii Vanbreuseghem 1952, have been studied since the latter was described by Vanbreuseghem (1953). Histoplasma capsulatum, a parasite of man and animals occurs in many countries while H. duboisii is apparently restricted to the African continent. In an extensive literature review of the comparative morphology and immunology of these two species Ajello (1968) concluded that H. duboisii should be referred to as H. capsulatum Darling, 1906 var. duboisii (Vanbreuseghem, 1952) Ciferri, 1960.

Many attempts have been made over the last 15 years to differentiate H. duboisii from H. capsulatum, but all of these have shown few distinct differences. The only certain way, up to the present time to separate these two species, is by animal inoculation to demonstrate the larger yeast form typical of H. duboisii (Blumer and Kaufman, 1968). Surpisingly, within the same strain (isolate) of H. capsulatum, great variations were found in the microscopic and macroscopic morphology of the mycelial phase in a primary subculture from a patient. Berliner (1968) gave the name type "A" to the organisms

that formed white cottony colonies having broad hyphae and few macroconidia and type "B" to the brownish colonies having narrow hyphae and numerous tuberculate chlamydospores. Thus, the species of H. capsulatum itself seems to be composed of a heterogeneous group of variants.

The objectives of this research are to further study the differences and similarities in H. capsulatum type "A", type "B", and H. duboisii by means of disc electrophoresis, enzyme assays through the use of electron microscopy, and by development of a differential medium. A comparison of the protein pattern and certain extracellular enzymes that are produced by these organisms in the mycelial and yeast phase may aid in a definitive separation of H. duboisii from H. capsulatum or support the proposed combination of H. duboisii into H. capsulatum Darling 1906 var. duboisii (Vanbreuseghem, 1952) Ciferri, 1960.

Any added knowledge concerning the differentiation of these two closely related organisms would be especially helpful in a diagnostic laboratory.

REVIEW OF LITERATURE

Histoplasma capsulatum was first discovered by Darling (1906). This organism, studied in detail by De Monbreun (1934), Howell (1939), Conant (1941), Dowding (1948), Pine (1960) and Goos (1964), is a dimorphic fungus capable of transforming into yeast-like cells, usually 2-4μ in diameter, when grown in animal tissue or on blood agar medium at 37 C and mycelial form on Sabouraud glucose medium at 25 C. This fungus is a human pathogen, the causative agent of histoplasmosis. The parasitic yeast-like form is found in tissue, while the mycelial form growing in Sabouraud glucose agar produces a white cottony colony which later becomes brown or tan in color. Microscopically, it has septate branching hyphae, 1.5-2.5μ thick. On these hyphae characteristic tuberculate, thick wall chlamydospores of variable sizes are produced. Based on microscopic and macroscopic morphology of the mycelial phase of primary isolates and their subcultures, Berliner (1968) found that there were two types of colonies. Type "A" was designated for albino type colonies, characterized by producing white, cottony broad aerial hyphae. Macroconidia are smooth and not numerous while microconidia are smooth or spiny.

Diffusible pigment may be seen after the colony is at least one month old. This type fails to produce spores on modified Sabouraud agar. In type "B" or brown form, the colony is flat becoming light tan to dark brown within a few days. Hyphae are narrow and pigmented with enormous numbers of tuberculated macroconidia which are characteristic of the species. Rapid development of diffusible brown pigment may be seen in the agar medium. The primary colony, from which type "A" and "B" are derived, is named type "P" for parent which consists of a mixture of type "A" and "B", and eventually type "A" overgrows type "B". Several varieties of spore shapes and sizes were found in type "P". The macroconidia of both types are usually borne singly on simple or branched conidiophores but may be in chains or so attached as to suggest budding. When these three types are converted to yeast-like phase, they are microscopically and macroscopically indistinguishable. The virulence of type "A", "B" and "P" are different (Daniels, Berliner and Campbell, 1968). When the same amount of yeast-like cells of type "A", "B" and "P" are injected intravenously into rabbits, animals receiving type "P" and "B" yeast cells died within 10 to 14 weeks. Those with type "A" survive considerably longer or recover.

Conant (1941) studied the life cycle of H. capsulatum. He cultured the mycelial form of the fungus in sealed blood agar tubes at 37 C. After 10-14 days, the yeast-like form of the fungus was recovered. He also demonstrated the development of the mycelial form of H. capsulatum from a yeast cell by using the slide culture technique and incubating the culture at 24 C.

Histoplasma duboisii Vanbreuseghem 1952, a species separated from H. capsulatum by Van breuseghem (1953) is the causative agent of a form of histoplasmosis distinguishable from the classical histoplasmosis of Darling by the relatively large intracellular parasite. This form of the disease occurs only in parts of tropical Africa, notably Nigeria, Ghana, the Belgian Congo and the French colonies of Senegal and Soudan (Duncan, 1958). This species was studied in detail by Vanbreuseghem (1953) and Duncan (1958). From their reports, the agent of African histoplasmosis is a dimorphic fungus, similar to H. capsulatum except the parasitic yeast form of H. duboisii is larger, measuring 8-12μ and occasionally up to 15μ in the longer axis. In the lymph nodes of patients, the cells were ovoid, measuring 10-13.5μ. They had thick double contoured walls and contained very large amounts of fat in the form of single round or smaller globules. Vanbreuseghem (1953) pointed out that the chlamydospores of H. duboisii were perfectly round while those of H. capsulatum were sometimes pyriform. This comment was argued by Drouhet (1962) as

he noted that cultures of H. duboisii formed oval and pyriform as well as spherical tuberculated chlamydospores on Sabouraud agar. Duncan (1958) found that large parasitic yeast cells of H. duboisii could only be obtained five months after inoculation into adult English mice, while Vanbreuseghem (1953) succeeded in obtaining these cells in two months after inoculation of guinea pig testes.

In culture media, Pine et al. (1964) found that strains of H. duboisii formed yeast cells of two sizes within a strain. One was similar to H. capsulatum, having a thin wall and containing no lipoidal globule, the other was larger and tended to develop large, thick-wall cells with lipid granule(s) or "duboisii form" (Vanbreuseghem, 1953). They also found that when the cultures were maintained at a young age by frequent sub-culturing, the cell population could be classified as being the same as H. capsulatum.

Because of the small size of the yeast-like cells of the organisms in the parasitic form as well as in cultures at 37 C, it is not possible with either the ordinary light or phase contrast microscope to obtain a precise picture of the cellular components. Edwards et al. (1959) studied the fine structures of the yeast phase of H. capsulatum and H. duboisii by electron microscopy using

90% butyl metacrylate and 10% methyl metacrylate as embedding material with 1.5% of lucidol as the initiator at 48°C for 36-40 hours. They reported that the capsule or slime layer on the outside of the cell wall of both species does not exist. The cell wall of H. duboisii is slightly thicker than H. capsulatum, but the cell structures of both species are similar, consisting of endomembrane systems, the same numbers and forms of mitochondria and the same nuclear fine structure. Apparently the first comparison of fine structure of the mycelial phase has been done by Garrison et al. (1970). They investigated the ultrastructural changes during the conversion of the yeast-like cells to mycelial phase of H. capsulatum and described some fine structure of the developing mycelium or primary hyphae from a yeast-like cell. They noted among the hyphal fine structure, intracytoplasmic membrane systems were in association with glycogen-like bodies. Woronin bodies (Reichel, 1965) were found near the septa. The ultrastructure of microconidia or chlamydospores of H. capsulatum was investigated by electron microscopy by Edwards, Hazen and Edwards (1960). They discovered that the tuberculate spore is the result of the thickening of the spore wall while the tubercle is a part of the cell wall formed by expansion of the outermost layer of the cell wall. Developing spores, according to Edward et al. (1960)

have various organels, endoplasmic reticulum, mitochondria, nucleus, microdroplets, irregular clumps of lipid and a cytoplasmic membrane. The mature spores have increasing amount of lipid, while the mitochondria and reticulum are invisible and the residual cytoplasmic matrix becomes homogenously granular.

As previously mentioned, the only way according to Blumer and Kaufman (1968) to separate H. duboisii from H. capsulatum is by animal inoculation. After two months the large yeast forms may be found in animal tissue for H. duboisii. Contradictary works have been also reported. Crumrine and Kessel (1931) noticed the large forms of H. capsulatum in the spleen of a patient. Drouhet and Schwarz (1956) have shown that large forms measuring 6 to 27μ, similar to the organism observed in African human and animal histoplasmosis were found when 12 American and 3 African strains were inoculated into hamsters. Large yeast forms could be induced by culturing the organism in blood agar base at 37C for 1 week as they grew from pieces of mice which had been inoculated intravenously 3-4 weeks previously with the mycelial phase of H. capsulatum (Schwarz, 1953). Binford (1955) reported from the study of 22 fatal cases of disseminated histoplasmosis and from lesions obtained surgically in non-disseminated cases that large yeast-like cells of H. capsulatum measuring up to 15μ were also found in necrotic tissue.

Many mycologists have tried to differentiate H. duboisii from H. capsulatum by biochemcial means but these organisms vary in their enzymatic reactions and their nutritional requirements so that no definite patterns in biochemical reactions can be used to differentiate these two organisms at present. Montemartini and Cifferi (1953) first reported that H. capsulatum utilized histidine and ammonium sulphate as nitrogen sources but H. duboisii did not. They used only a single strain from the two species, so their results need to be confirmed. Coremans (1963) reported that H. capsulatum produced urease in 24-48 hours while H. duboisii failed to do so after 48 hours, however, this could not be confirmed by Rosenthal and Sokolsky (1965) but they found that H. capsulatum hydrolyzed tyrosine, not gelatin while H. duboisii hydrolyzed gelatin but not tyrosine. These findings were more significant when Berliner (1967) confirmed that the mycelium of H. duboisii liquified 15% plain gelatin in 24-96 hours whereas H. capsulatum did not in 6 weeks. She used 70 strains of H. capsulatum from various sources and 7 strains of H. duboisii. Blumer and Kaufman (1968) could not confirm the work of Berliner nor all previous reports when they used 10 strains of H. capsulatum and H. duboisii. Nine of ten strains of H. duboisii

hydrolyzed 0.4% gelatin within 3 weeks, 6 strains hydrolyzed 15% gelatin in 96 hours, however, 5 strains of H. capsulatum were able to hydrolyze 0.4% gelatin and all 10 strains hydrolyzed 15% gelatin. Only 7 strains of H. capsulatum hydrolyzed tyrosine but 3 strains of H. duboisii did too. Three strains of H. duboisii were also found to produce urease within 48 hours on the solid media.

Although the two organisms are almost indistinguishable in vitro, striking difference in their clinical manifestations were pointed out by Olurin, Lucas and Oyediran (1969). They found H. capsulatum infections may cause (1) acute pulmonary and disseminated disease which is frequently fatal, or (2) a benign subclinical form leading to pulmonary calcification and a positive histoplasmin skin test. Histoplasma duboisii infections cause either (1) a localized form with solitary skin nodule, (2) bone lesion, or (3) a disseminated form involving skin, subcutaneous tissues, lymph nodes, bones, joints and abdominal viscera, but rarely lungs.

Most of the previous biochemical investigations included studies on production of enzymes by H. capsulatum and H. duboisii were detected by indicators such as phenol red for the detection of pH change in media, (urease test; sugar fermentation media), and hydrolysis of the media (gelatin liquefaction, casein hydrolysis and

etc.). Enzyme production in fungi has been found to be affected by nutritional and environmental factors (Wilson and Niederpruem, 1967). Since strains of H. capsulatum differ in vitamin and amino acid requirements (Rowley and Pine, 1955), enzyme production should be studied in a medium rich in vitamins and amino acids. Beneke, Wilson and Rogers (1969) used both liquid and solid casein-peptone-yeast extract-glucose (2.0 g, 2.0 g, 2.0 g and 5.0 g respectively/500 ml. water) media for study of enzymatic activities of Blastomyces dermatitidis. After 5, 10, and 15 days of incubation, the liquid filtrate and agar medium of the yeast and mycelial phases were assayed for enzymatic activities. High activities of paranitrophenyl acid and alkaline phosphatases were found in both media for the yeast phase in comparison to the mycelial phase.

Regarding the antigenic structures of H. capsulatum and H. duboisii, Drouhet (1967) studied the antigenic structures of these two organisms. He used antigens prepared from lyophilized histoplasmin and obtained the sera from rabbits hyperimmunized with

ground mycelium. By the use of agar double diffusion technique, he found at least four common fractions in the two types of histoplasma and two fractions specific for H. capsulatum. With immunoelectrophoresis, the serum from ground mycelium hyperimmunized rabbit showed at least eight antigenic fractions in the histoplasmin of H. capsulatum and five or six fractions in the histoplasmin of H. duboisii. Two specific fractions of H. capsulatum were also demonstrated by exhausting the immune serum with heterologous antigens. Andrieu et al. (1969) also applied agar gel diffusion and immunoelectrophoresis in their comparative study of H. capsulatum and H. duboisii, but the results were not identical with that of Drouhet. They concluded that antigenic structures of these two organisms were the same, at least qualitatively.

Kaufman and Blumer (1961) studied extensively the antigenic structures of 56 strains of H. capsulatum and 11 strains of H. duboisii by fluorescent antibody specific for the yeast phase of H. capsulatum by means of adsorbed labeled antisera with cells of known antigenic structures and staining titers. All 56 strains of H. capsulatum tested were subdivided into 5 serotypes represented by the combination of numbers 1, 2; 1, 4; 1, 2, 3; 1, 2, 4; 1, 2, 3, 4. One serotype 1,4 was closely related antigenically to H. duboisii and Blastomyces dermatitidis. These discoveries later

resulted in the development by Kaufman and Blumer (1968) of a polyvalent conjugate to differentiate Histoplasma from other organisms but this conjugate failed to separate H. duboisii from H. capsulatum.

When Pine (1970) analysed the yeast phase cell wall amino acids of H. capsulatum serotype 1,4 and H. duboisii, he found that the relative molar concentrations of the individual amino acids were similar with a higher threonine content in H. duboisii. He also discovered that the amino acids in the mycelial cell walls of the two types of Histoplasma were not significantly different.

Leone (1964) has reported that protein electrophoresis can be used as a valuable tool for the study of phylogenetic relationships, as protein structure is primarily an expression of the genotype of an organism. Since species may be determined by their genotypes, the study of proteins by electrophoresis is important in identifying of the species. Characteristic of electrophoretic mobilities which can be demonstrated by protein staining is unique and reproducible for a particular species. Disc electrophoresis (Ornstein and Davis, 1962) is the sensitive technique used to separate protein components by means of a charge and molecular sieving. It is a highly reproducible method for the study of proteins (Whipple, 1966). This technique has been applied to taxonomic studies of

several microorganisms. Chang et al. (1962) demonstrated the difference among species of Neurospora by electrophoretic fractions of soluble protein extracted from mycelium by disc electrophoresis on polyacrylamide gels. They also showed that a mutant strain of N. crassa possessed a number of slow-moving bands not present in the pattern of the wild type strain used. A similar report by Clare (1963) has demonstrated that Pythium species have the same protein pattern even when isolated from different locations. Durbin (1966) in his investigation of electrophoretic patterns of many isolates of Septoria, found that the majority of the patterns were similar among strains in the same species although variation in patterns within each species also existed. The complexity of dermatophytes has been clarified by Shechter et al. (1966) by the application of disc electrophoresis of the proteins extracted from mycelial mats or culture filtrate; each species has specific electrophoretic pattern and has common fractions in the same genus. Furthermore, Shechter, Landau and Newcomer (1967) found that culture filtrates of Coccidioides immitis from different media or the same media at different incubation periods yielded different protein patterns by disc electrophoresis. Thus, if H. capsulatum and H. duboisii are grown in the same media with the same amount of inocula, and under the

same environmental conditions, the culture filtrates of both organisms should yield characteristic protein patterns by disc electrophoresis.

Recently, Rottem and Razin (1967) proposed that electrophoretic patterns of membrane proteins be used as finger prints of Mycoplasma and later, they recommended the same method he used to identify other microorganisms (Razin and Rottem, 1967). Since the phenol-acetic acid-water was found to be very active in solubilizing the structural membrane proteins of the cells (Takayama et al., 1966), it might be used to dissolve membrane proteins of Histoplasma and the protein solution could be subjected to disc electrophoresis. Since it is believed that the synthesis of membrane proteins is directed in part by heredity (Korn, 1966), the electrophoretic patterns of membrane proteins of the two types of Histoplasma may reflect phylogenetic relationships.

MATERIALS AND METHODS

Disc Electrophoresis of Soluble Protein from Cultures of Histoplasma capsulatum and Histoplasma duboisii

Extraction of Soluble Protein

Histoplasma capsulatum strain 184 G type "A" and type "B" obtained from Berliner, Harvard University, and *Histoplasma duboisii* from the M.S.U. stock culture collection were grown in 250 ml Erlenmeyer flasks with 100 mls of liquid medium consisting of 0.4 gm. each of casein, neopeptone, yeast extract and 1.0 gm. of glucose (CPYG medium). The inoculum consisted of approximately 1800×10^6 yeast cells per flask from a 6 day culture which was growing on solid medium of the same composition. Each organism was cultured in triplicate and incubated at 25 C and at 37 C on rotary shakers at 160 rpm. At the end of 10, 20, and 30 days of incubation, one culture from each group of flasks was checked microscopically for contamination and then filtered through seitz sterilizing filters. Two volumes of cold acetone were added gradually into one volume of culture filtrate and uninoculated broth, and stirred gently. The acetone broth filtrate mixtures were left for several hours in the cold room. Protein precipitate was then removed by centrifugation at 18,000 g for 15

minutes and redissolved in distilled water. Protein determinations of the solutions were performed by Lowry's method (Lowry, et al. 1951). The protein extracts were kept frozen at -20 C for periods up to 4 weeks.

Disc Electrophoresis Procedure

The method for disc electrophoreses as described by Davis (1964) was adopted. Polyacrylamide gel columns were prepared in 5 x 100 mm. cylindrical glass tubes. The gels were composed of two layers. The upper layer having the total length of 1 cm, was the large pore gel consisting of 2.8% acrylamide, N,N,N',N', tetramethylethylenediamine, N,N' methylenebisacrylamide, riboflavin, Tris-HCl and water. The lower layer with a total length of 6.5 cm. was the small pore gel in which electrophoretic separation took place. It consisted of 7.5% acrylamide, N,N,N',N', tetramethylethylenediamine, N,N' methylene bisacrylamide, ammoniumpersulfate and buffering systems. For convenience, 6 stock solutions had been previously prepared as follows:

Stock A		Stock B	
1 N HCl	48 ml.	1 N HCl	approximately 48 ml.
TRIS	36.6 g.	TRIS	5.98 g.
TEMED	0.23 ml	TEMED	0.46 ml
Water to	100 ml	Water to	100 ml
	pH 8.9		pH 6.7

Stock C		Stock D	
Acrylamide	28.0 g	Acrylamide	10.0 g
BIS	0.735 g	BIS	2.5 g
Water to	100 ml	Water to	100 ml

Stock E	Stock F
Riboflavin 4 mg	Sucrose 40 g
Water to 100 ml	Water to 100 ml

For the preparation of the lower gel, a solution of 1 part A, 2 parts C, 1 part water, 4 parts small pore solution (0.15 g ammonium persulfate in 100 ml water) was made and then poured into an upright cylindrical glass tube by a capillary pipette up to 6.5 cm high. Before polymerization, the upper top surface was flattened by a thin layer of water. The solution of the upper gel was a mixture of 1 part B, 2 parts D, 1 part E and 3 parts F. It was layered 1 cm high over the lower gel after the water had been drained away.

For operation of electrophoresis, 300-400μ g of soluble protein extract was dissolved in 0.2 ml of 40% sucrose solution and carefully layered on top of the large pore gel. The acrylamide columns connected the upper and lower jars which were filled with tris-glycine buffer at pH 8.6 Direct current of 200-350 volt and 5 milliamperes per gel were connected with the cathode on the upper jar and the anode at the lower jar. Both electrodes were immersed in the tris-glycine buffer. In the upper buffer, a few crystals of bromphenyl blue were dissolved as tracking dye. The protein samples were concentrated in the upper gels; moved and separated in the lower gels. The front boundary, as seen by tracking dye, was allowed to move down for 5 cm from the upper gel. The gels were

removed from the glass tubes and cut away at the tracking dye. They were stained by Buffalo Black (0.5% in 7% acetic acid) for one hour and destained by submerging in 7% acetic acid overnight.

Disc Electrophoresis of Protein Extract from the Culture Filtrates of the Mycelial Phase of Additional Strains of H. capsulatum and H. duboisii

After 3 to 4 weeks of growth on Sabouraud dextrose agar at 25 C, mycelial mass and spores of each strain were scraped from an agar slant into 4 ml of sterile physiological saline. Four ml were inoculated to 100 ml of CPYG broth in 250 ml flask and incubated on a rotary shaker for 30 days at 25 C. After checking for contamination, microscopically and culturally, the liquid medium was filtered through seitz filters. Protein extraction, protein determination and disc electrophoresis was performed as previously described.

Disc Electrophoresis of Phenol Acetic Acid Water Cell Extracts

Ten day-old cultures of Histoplasma capsulatum G-184 type "A" and "B", and H. duboisii grown on CPYG agar slants at 37 C were scraped into physiological saline, centrifuged and washed with saline solution again. One milliliter of phenol-acetic acid-water (2:1:0.5 w/v/v) was added to 150 mg of wet weight of the organism. After the mixtures were left overnight at 4 C then, the insoluble materials were removed by centrifugation at

30,000 g for 15 minutes. One tenth of the clear supernatant was mixed with 0.05 ml of a 40% (w/v) sucrose solution in 35% (v/v) acetic acid. The sample sucrose mixtures were layered on top of gels for electrophoresis.

The same procedure was used for 10 day-old yeast phase broth cultures of different strains of H. duboisii and H. capsulatum. A modification of Razin and Rottem (1967) method for the acrylamide gel was made. In preparation of the gels, stock solution of A and B were made as follows:

Solution A		Solution B	
Acrylamide gel	3.5 g	Urea	6 g
Urea	6 g	Ammonium persulfate	0.15 g
N,N'methylbisacrylamide	0.08 g	Water to 10 ml	
47% acetic acid v/v to 30 ml			

Three volumes of solution A were mixed with one volume of solution B, then 0.02 volume of N,N,N'N' tetramethylethylenediamine was added to the solution. The final mixture was put into 100 x 5 mm cylindrical glass tubes and overlayered with 75% (v/v) acetic acid. Polymerization of acrylamide gel was carried out at 37 C for 45 minutes.

After the sample sucrose mixtures had been put on top of the gels, 0.5 ml of a 75% acetic acid was carefully layered on top. Both upper and lower jars of the electrophoretic apparatus were filled with 10% (v/v)

acetic acid. The lower electrode was cathode with direct current of 5 milliamperes per tube. The front boundary was allowed to move down for 5.5 cm with approximately 70 minutes of running time. The gels were taken out of the glass tubes and stained with 0.5% (w/v) Buffalo Black in 7% acetic acid. After 1 hour of staining, the gels were destained by submerging in 7% acid (v/v) overnight.

Enzyme Activities

Survey for Enzymatic Activities in Three Kinds of Liquid Media at 37°C and 25°C

Yeast phases of Histoplasma duboisii and H. capsulatum strain G-184 type "A" and "B" in CPYG agar slants were transferred to 100 ml of each of the following: Sabouraud dextrose broth; brain heart infusion with 0.1% cysteine; and CPYG broth. Each inoculum consisted of 2 ml of approximately 900 x 10^6 cells per ml. One set of cultures in 250 ml flasks was

incubated on a rotary shaker at 37 C, the other, at 25 C. Cultures in Sabouraud dextrose broth were incubated at 25 C, only. At the end of 10, 20, and 30 days of incubation a 10 ml portion from each culture was filtered through a seitz sterilizing filter. The culture filtrates were used as the enzyme sources to assay for enzymatic activities. The following chemicals were used as substrates: paranitrophenol-alpha D-glucopyranoside (1.0 mg/ml in sodium acetate buffer, pH 5.4), PNP-beta-D-glucopyranoside (1.0 mg/ml in sodium acetate buffer, pH 5.4), PNP-alpha-D-galactoside (1.0 mg/ml in sodium citrate-phosphate buffer, pH 7.0), PNP-beta-D-galactoside (1.0 mg/ml in citrate-phosphate buffer, pH 7.0, PNP-N-acetyl-beta-glucosaminide (1.0 mg/ml in sodium acetate buffer, pH 5.4), PNP-beta-D-glucuronide (1.0 mg/ml in sodium acetate buffer, pH 4.5), PNP-phosphate (1.0 mg/ml in Tris-HCl buffer pH 8.6 for alkaline phosphatase, and in sodium acetate buffer, pH 5.2 for acid phosphatase). The concentration of each buffer was 0.1 M. Nine-tenth ml of each substrate solution was transferred into test tubes, 0.2 ml of culture filtrate was added into each tube of substrates, mixed well and incubated at 37 C for two hours. Enzymatic reactions were stopped by adding 2.0 ml of 1.0 M Tris pH 9.8. The activity of the enzymes was proportional to the yellow color of paranitrophenol released from the substrates which was measured with a spectrophotometer at

410 mμ (Turner, model 330). Enzymatic activities were expressed as optical density per milliliter of broth filtrate per one hour and multiplied by 1000.

PNP-alpha and beta glucosidase were found to be greatest in the CPYG culture filtrate of H. duboisii at 37 C. On this basis, filtrates of cultures between 10 to 30 days of incubation were used to investigate some characteristics of these enzymes.

Some Characteristics of PNP-Alpha and Beta Glucosidase

Enzyme Production in Relation to the Age of Culture in Liquid Media at 37 C.--A ten day-old culture of H. duboisii in liquid CPYG at 37 C containing approximately 829 x 10^6 cells per ml was used as inoculum. Two mls of this inoculatum were inoculated into 100 ml of the same medium in 250 ml flasks and incubated on a rotary shaker at 37 C. Each day over a ten day period, total cell counts and enzymatic activities for PNP-alpha and beta glucosidase were determined and recorded. This was continued periodically over the entire 30 day period.

Enzyme Production in Relation to the Age of Culture on Solid Medium.--Six day-old cultures of the H. duboisii growing on CPYG agar plates were used as the inocula. The organism was heavily streaked in a 9 square cm area on CPYG agar and incubated at 37 C.

Small plugs of agar medium near the edges of the growing colonies were removed for testing enzyme activity. A cork borer was used to insure that constant amounts of medium were taken for each sample and 0.9 ml of substrate was added per plug of agar. The enzymatic reaction was stopped by additional 2 ml of 1.0 M Tris after 1 hour of incubation at 37 C. Enzymatic activities of the organisms were recorded at various times during the 30 day incubation period.

Optimal Temperature and Optimal pH.--Nine tenth ml of PNP-alpha and beta glucopyranoside in sodium acetate buffer pH 5.4 and 0.1 ml of broth filtrate were mixed and incubated at 4 C, 25 C, 59 C, 64 C, 69 C and 75 C for 1 hour. Activities were recorded. Enzyme activities were also checked after 3 minutes in the steamer.

Substrate (PNP-alpha and beta glucopyranoside) were dissolved in Tris-citrate-phosphate buffer (1 mg/ml) in ranges of pH from 3.0 to 9.0. From a preliminary test, the optimal pH of both enzymes from H. duboisii for the highest activities were between pH 4.0 to 5.0. For a more critical determination of pH, the buffers were adjusted to the following pH values: 4.2; 4.4; 4.6; 4.8; 5.0 and 5.3. Substrates were dissolved in buffer (1 mg/ml) at each pH, and 0.1 ml of broth filtrate for each 0.9 ml substrate for the various pH values was

used for assay. The enzyme activities at the different pH readings were recorded.

Effect of Storage at -20 C.--Enzyme activities were first investigated on the filtrate before storing in the freezer. After a period of 90 days of storage in the freezer at -20 C, the enzyme activities were rechecked. The assay method was the same as previously described.

Investigation of the Kinetics.--A similar method of enzyme assay for PNP-alpha and beta glucosidase was used with varied times of incubation at 0, 5, 10, 15, 25, 40, 60, 90, 212 minutes. The rise of enzyme activities in comparison to the time factor were plotted graphically.

Inhibition Test.--The following sugars: glucose, mannose, galactose and d-levulose were tested as possible inhibitors of PNP-alpha-and-beta-glucosidase in the concentration of 0.46 M, 0.09 M, and 0.04 M. The substrate concentration was 1 mg/ml in sodium acetate buffer pH 5.4. One tenth ml of broth filtrate from H. duboisii was used as the enzyme source. The reaction temperature was 37 C for 1 hour. The inhibiting effects were calculated as percentage activity over control (water was used as a control to replace inhibitors).

Effects on Enzyme Production due to the Modifications in Liquid CPYG Medium.--

a. Casein and the amount of glucose.

The glucose in the CPYG medium was varied in amounts from 0, 0.5%, 1.0%, 1.5% to 2.0%. Another test medium was CPYG, but vitamin free casein was replaced by casamino acids in the same proportion. Two ml of a 10 day-old culture of H. duboisii in CPYG broth (casein was replaced by casamino acids) having approximately 828.7 x 10^6 cells/ml was inoculated into 250 ml flasks containing 100 ml of test medium and incubated on a rotary shaker at 37 C. After 15 days, all cultures in the test medium were filtered through seitz sterilizing filter. The broth filtrate of each culture was assayed for activities of PNP-alpha and beta glucosidases by the same method as previously described.

b. Sugars and Carbohydrates.

The glucose in CYPG medium was replaced by either d-lactose, d-cellobiose, soluble starch, or dextrin, in the same amount by weight. Two ml of a 4 day-old culture of H. duboisii in CPY broth containing approximately 60.4 x 10^6 cells/ml was inoculated into 100 ml of the test media in 250 ml flasks and incubated on a rotary shaker at

37 C for 15 days. Activities of PNP-alpha and beta glucosidases were assayed by the method given above.

Identification of PNP-alpha and Beta-glucosidases Fraction of Lyophilized Culture Filtrates of H. duboisii in CPYG Broth at 37 C by Disc Electrophoresis.--Histoplasma duboisii was cultured for 30 days in 100 ml of CPYG broth inoculated with 1 ml of a cell suspension containing approximately 900 x 10^6 cells ml from a six day old culture on CPYG agar. The broth filtrate was lyophilized and used as the protein source for disc electrophoresis. Six milligrams of lyophilized material was dissolved in 0.2 ml of 40% (W/V) sucrose solution and put on top of the large pore gels. Electrophoresis of this material was performed in triplicate. One of the gels was stained with Buffalo Black for 1 hour and destained in 7% acetic acid electrophoretically. While the first gel was staining, the other two were put in test tubes, one containing PNP-alpha-D-glucopyranoside the other containing PNP-beta-D-glucopyranoside, for 1 hour. The substrates were replaced by 1.0 M Tris. The yellow bands of paranitrophenol were visible immediately, indicating the location of PNP-alpha and beta glucosidase. The position of the yellow bands were compared with the homologous bands that stained with Buffalo Black.

Enzymatic Activities of PNP-alpha and Beta Glucosidases in the Mycelial Phase of Other Isolates of Histoplasma Capsulatum and H. duboisii.--Two more isolates of H. duboisii strain B 937 and B 939 and three isolates of H. capsulatum strain B 621 from soil, B 293 from human lung, B 815 from dog, were obtained from Dr. B. Georg, Communication Disease Center, Atlanta Ga. Four isolates of H. capsulatum strain 186-G type "A" and "B", strain 219 type "A" and "B" were obtained from Berliner, Harvard University. Three isolates of H. capsulatum strain D-213, 68-46 and 488 were obtained from Dr. Furcolow, University of Kentucky. All isolates were cultured on Sabouraud dextrose agar in plates at room temperature (25 C). After 30 days, a portion of the agar medium near the edge of the colonies was assayed for PNP-alpha and beta glucosidase using the agar disc method as previously described.

The Distribution of PNP-alkaline Phosphatases in Polyacrylamide Gels from Disc Electrophoresis of Lyophilized Culture Filtrate from Strains of H. capsulatum and H. duboisii in CPYG Broth at 25 C

Six milligrams per gel of lyophilized culture filtrate of H. capsulatum strain 184 G type B, 186 G type B, 219 type B and H. duboisii MSU strain, B 937, B 939 were fractionated by disc electrophoresis as previously described. The gels were then, cut into

sections 0.5 cm long and assayed for alk-PNPPase activity in each section. One ml of 1 mg/ml of PNP-phosphate in Tris-HCl buffer at pH 8.6 was added to each section of the gel and incubated at 37 C for two hours. Reactions were stopped by adding 2.5 ml of a 1.0 M Tris. The activities were measured by a spectrophotometer (Turner, Model 330) at 410 mμ.

A Study of Alpha and Beta-PNPGases and Alkaline-PNPPase in Histoplasma Capsulatum Type "A" and "B"

Three strains of H. capsulatum type "A" and "B" were grown on BHI agar with 0.1% cysteine and incubated at room temperature (25 C) for 20 days. A portion of agar medium near the edge of the colony of each culture was taken with a cork borer of 0.5 cm in diameter to use as the enzyme sources. Nine tenth ml from each of the substrates (PNP-alpha and beta-D glucopyranosides 1 mg/ml in 0.05 M acetate buffer pH 5.4 and PNP-alkaline phosphate 1 mg/ml in 0.1 M Tris-HCL buffer pH 8.6) was added to a plug of agar medium to detect enzyme activity in solid medium cultures. The procedure continued the same as previous enzyme experiments.

Study of Alpha and Beta-PNPGases and Alkaline-PNPPase Activities in Five Strains of H. capsulatum and H. duboisii in CPYG Agar Plates for 9 days at 37 C

In order to confirm the differences in the three enzyme productions on CPYG agar plates at 37 C of these two organisms. Five strains of the yeast phase of H. capsulatum strain B 913, B 329, 105, MSU, 222A and H. duboisii strain B 650, B 651, B 652, B 939, MSU were grown in CPYG agar plates to study the enzymes in the agar medium. The method was the same as previously described.

Comparison of Ultrastructure of Histoplasma Capsulatum and H. duboisii

Histoplasma capsulatum and H. duboisii were grown in 100 ml Sabouraud dextrose broth in 250 ml flasks on a rotary shaker at room temperature (25 C) for ten days. The small pellets of mycelium were filtered out and washed with phosphate buffer pH 7.4 and fixed with 3% glutaraldehyde in phosphate buffer pH 7.4 for 2 hours. Then, the glutaraldehyde was drained away, the pellets were washed 3 times with phosphate buffer. The pellets were later postfixed with 1% osmium tetroxide in phosphate buffer for 1 hour. The specimens were dehydrated with 25%, 50%, 75%, 95% ethyl alcohol for 10 minutes each and 100% ethyl alcohol for 20 minutes twice. After dehydration, propylene oxide was added to

the specimens twice for 30 minutes each. Then, propylene oxide was replaced by propylene oxide plus EPON mixture (7 parts A and 3 parts B) with the proportion of lv/lv and left overnight.

EPON Mixture A		EPON Mixture B	
EPON 812	62 ml	EPON 812	100 ml
Dodecenyl succinic anhydride	100 ml	Methyl nadic anhydride	89 ml

Then the specimens were ready to embed in EPON complete mixture. Bean capsules were filled with EPON mixture first, then the specimens that had been submerged in EPON mixture plus propylene oxide overnight were picked up by a capillary pipette, released and pushed into the bottom of the capsules. The capsules with specimens at the bottoms were left overnight in a dissicator for complete penetration, then, put in 60 C oven for 2-3 days for hardening. Sections were cut on a Reichert ultramicrotome, mounted on 400 mesh copper screens, 3.0 mm in diameter, and observed in the Zeiss EM9A transmission electron microscope.

Attempts to Develop a Differential Medium for Histoplasma Capsulatum and H. duboisii

As a result of the investigation of the effect of nutritional factors on the activity of alpha and beta-PNP-glucosidase a modification of CPYG medium was made in an attempt to demonstrate the difference in color reaction in the colonies of H. capsulatum and H. duboisii. Three sets of CPYG agar media were prepared. The first

set of media contained two different concentrations of ferrous sulfate (0.2 g/l and 0.4 g/l). The second set contained two concentrations of the combination of ferrous sulfate and sodium thiosulfate (0.2 g/l and 0.4 g/l; 0.4 g/l and 0.5 g/l respectively). The third set contained one concentration of the combination of ferrous sulfate and cysteine (0.4 g/l and 0.5 g/l respectively). In the same way, three sets of modified BHI agar were prepared. All media were sterilized by autoclaving for 15 minutes at 121 C under pressure of 15 pounds/square inch. Ten day old cultures of 7 strains of H. capsulatum, G-184 A, G-184 B, G-186 A, G-186 B, 222 A, B 621, B 875 and 1 strain of H. duboisii in CPYG agar were inoculated heavily on these media and incubated at 37 C. The colonies of both species on each plate were observed periodically for the difference in color. Later, 4 additional strains of H. duboisii were used.

An indicator medium was prepared to observe the changes in the pH in the medium during the growth of H. capsulatum and H. duboisii strains for possible differentiation of the species. The medium had the following composition: casaminoacids; neopeptone, 2 g/l; yeast extract, 2 g/l; cellobiose, 10 g/l; phenol red, 0.05 g/l. The pH was adjusted to 5.1 with HCl. Histoplasma capsulatum strains 488, B-621, B-875, B-293, and H. duboisii strain B-937, B-938, B-939, and MSU were used. All cultures were inoculated with mycelial phase, and grown on slants

at 25 C. The color changes from yellow to red were noted periodically over a 9 day period.

RESULTS

Disc Electrophoresis of Soluble Protein Extracted from the Culture Filtrates of H. capsulatum Strain G-184 Type "A" and "B" and H. duboisii MSU Strain

Comparison of electrophoresis patterns of soluble protein from the broth filtrates of a 10 day culture at 25 C is shown in Figure 1. Only one clear band at rf 43.63 was demonstrable in H. capsulatum type "A" and "B". Histoplasma duboisii MSU strain had 2 fractions, one with the same rf as in type "A" and "B", the other had one at rf 21.81. All three organisms produced good mycelial growth in the culture medium. After 20 days in culture, H. capsulatum type "A" still produced only 1 fraction as in the 10 day culture but type "B" produced 8 fractions, 4 of which were very faint, located at higher rfs (60.0, 65.45, 74.54, 80.0). Histoplasma duboisii produced 4 fractions (23.63, 41.81, 45.45, 65.45). All of these are common to H. capsulatum type "B". Comparative electrophoretic patterns for 20 day culture is illustrated in Figure 2. In 30 day cultures, the protein fractions were most clearly separated. Two more light bands (rf 40.00, 54.54) were visible in H. capsulatum type "A". Seven fractions were produced in H. capsulatum type "B".

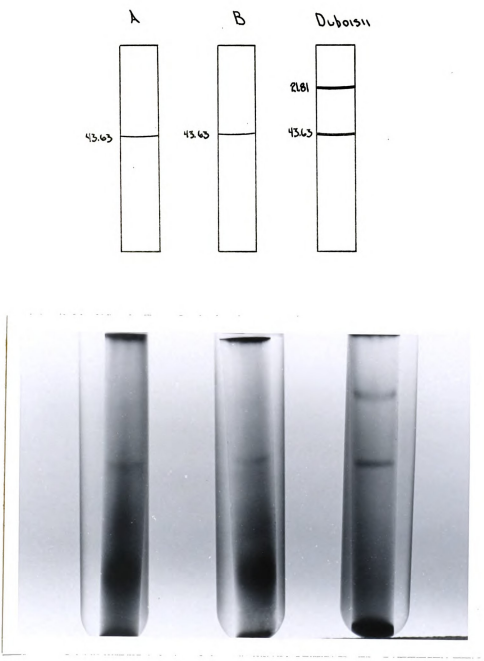
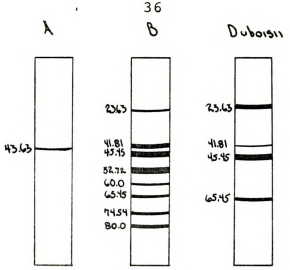
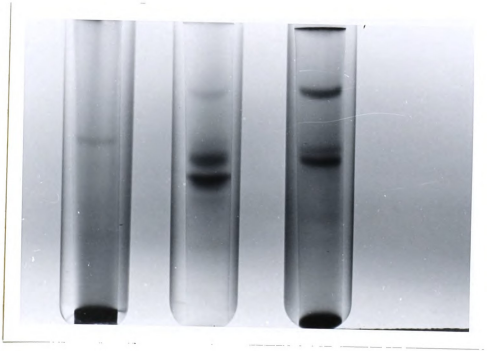


FIGURE 1.--Electrophoresis patterns of protein extract from culture filtrate of H. capsulatum strain G-184 type "A" and "B" and a strain of H. duboisii in CPYG medium for 10 days at 25 C.





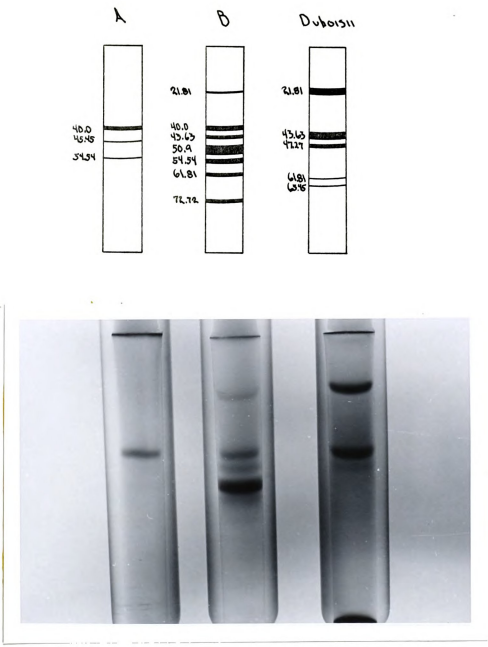
A B Duboisii

FIGURE 2.--Electrophoretic patterns of protein extract from culture filtrate of H. capsulatum type "A", type "B" and H. duboisii in CPYG medium for 20 days at 25°C.

Four of the protein bands (rf 21.81, 40.0, 43.63, 50.9) were intensely stained while those at rf 54.54, 61.81, 72.72 were very light. The fractions at rf 40.0, 43-45.45, 54.54 were common between type "A" and type "B" of this strain. Histoplasma duboisii had 5 fractions, 3 of which were clearly visible (rf 21.81, 43.63, 47.27). Three bands (21.81, 43.63 and 61.81) were common with type "B". Figure 3 summarized these results. The yeast phase cultures were incubated for 30 days at 37 C. The protein extracted from the culture filtrate of H. capsulatum strain G-184, type "A" produced 5 fractions (rf 18.18, 27.27, 52.72, 65.45, 81.81). For the same strain, type "B" produced only 2 fractions (rf 18.18, 52.72) which were homologous for the two types. Histoplasma duboisii showed one fraction (rf 41.81) intensely stained, the other two bands (rf 47.27, 52.72) were very faint. The fraction at 52.72 appeared to be common for all three isolates. The diagram of protein patterns is shown in Figure 4.

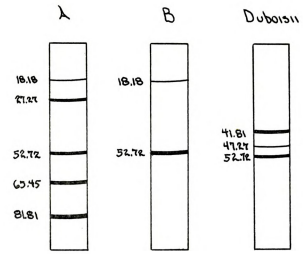
Disc Electrophoresis of Penol-Acetic Acid-Water Cell Extracts

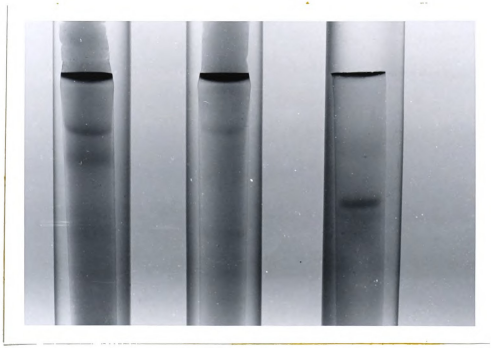
Cells, from 10 day old CPYG agar cultures at 37 C were extracted by the Phenol-acetic acid-water method for disc electrophoresis. Three isolates of H. capsulatum strain G-184 type "A" and "B" and a strain from our MSU stock culture, had almost identical patterns while



A B Duboisii

FIGURE 3.--Electrophoretic patterns of protein extract from culture filtrate of H. capsulatum type "A", type "B" and H. duboisii in CPYG medium for 30 days at 25°C.





A B Duboisii

FIGURE 4.--Electrophoretic patterns of protein extract from culture filtrates of H. capsulatum type "A", type "B" and H. duboisii in CPYG medium for 30 days at 37 C.

H. duboisii had similar positions of protein bands but some of them were more intensely stained. Both species produced such compact patterns of 8 to 10 protein bands that their rf values were difficult to calculate accurately. The photograph of the gels is illustrated in Figure 5.

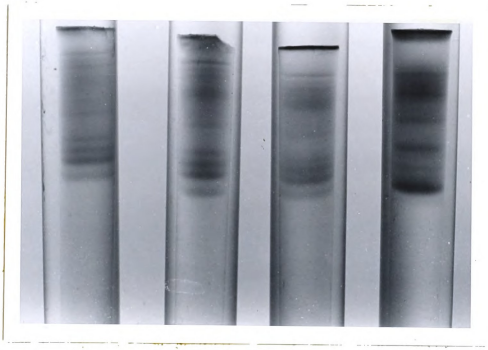
Disc Electrophoresis of Cell Extracts from the Yeast Phase of Additional Strains of H. capsulatum and H. duboisii Grown on CPYG Agar at 37 C

Protein extracts from the yeast cells of H. capsulatum, strain G-186 type "A", strain B-621, B 913, B 329, 105, strain 222 type "A", and H. duboisii, strain B-939, B-650, B-651 and B 6 were subjected to disc electrophoresis by the method of Razin and Rottem (1967).

The electrophoretic patterns of all strains of both species appeared to be similar although some faint bands at the lower rf values were not clear as illustrated in Figure 6.

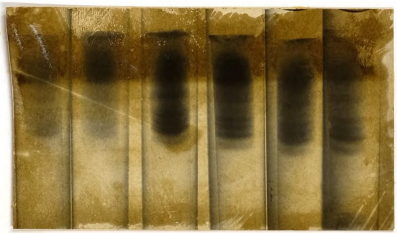
Disc Electrophoresis of Protein Extract from the Culture Filtrates of the Mycelial Phase of Additional Strains of H. capsulatum and H. duboisii

All strains grew very well in CPYG liquid medium at 25 C. The electrophoretic patterns of both H. capsulatum and H. duboisii appeared different, even

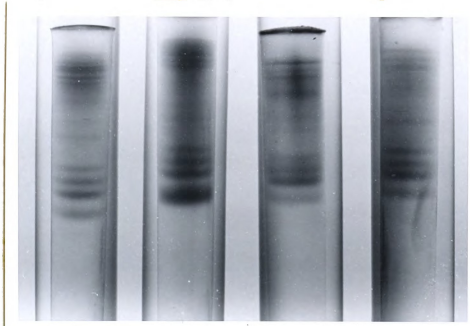


HC G184A HC G184B HC-MSU HD-MSU

FIGURE 5.--Electrophoretic patterns of protein extract from yeast cells of 3 isolates of H. capsulatum and 1 isolate of H. duboisii when grown on CPYG medium at 37°C.



B650 B651 B652 B329 B913 HC105



HC B621 HC 222A HC G186A HD B939

FIGURE 6.-- Photograph(s) of electrophoretic patterns of cell extracts from the yeast phase of additional strains of H. capsulatum and H. duboisii.

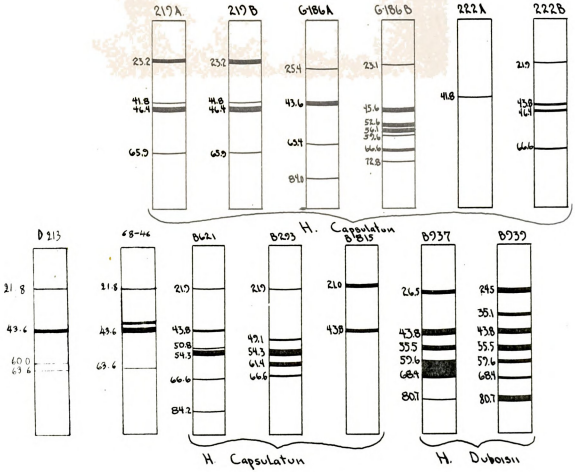
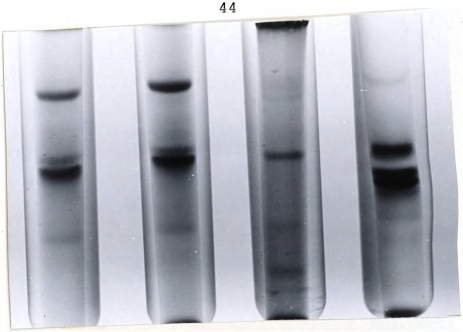
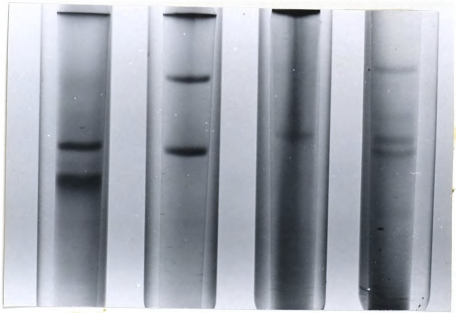


FIGURE 7a.--Diagrams of electrophoretic patterns in acrylamide gels of protein extracts from the culture filtrates of additional strains of H. capsulatum and H. duboisii in CPYG medium for 30 days at 25 C.

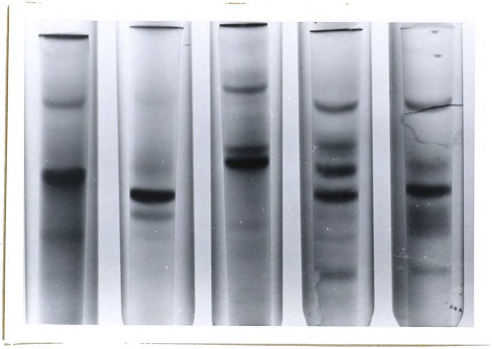


219A 219B G-186A G-186B



B621 B815 222A 222B

FIGURE 7b.--Photographs of electrophoretic patterns in acrylamide gels of protein extracts from the culture filtrates of additional strains of H. capsulatum and H. duboisii in CPYG medium for 30 days at 25 C.



D213 B293 68-46 B939 B-937

FIGURE 7c.--Photographs of electrophoretic patterns in acrylamide gels of protein extracts from the culture filtrates of additional strains of H. capsulatum and H. duboisii in CPYG medium for 30 days at 25 C.

strains in the same species were different. Moreover in the same strain with different types (Berliner, 1968) there was a variation. Only H. capsulatum, strain 219 type "A" and "B" showed the same pattern. However, some strains of H. duboisii had electrophoretic patterns which were more closely related than those in strains of H. capsulatum. For the two strains of H. duboisii, 6 fractions (rf 24.5, 43.8, 55.5, 59.6, 68.4, and 80.7) were in common. For 11 strains of H. capsulatum, 10 had one fraction (rf 21.0 - 23.2) in common. The fraction at rf 41.8 - 43.8 and 65.4 - 68.4 were believed to be homologous among seven strains of H. capsulatum and two strains of H. duboisii. The diagrams and photographs of these gels with protein bands are illustrated in Figure 7.

Enzyme Activities

Survey for Enzymatic Activities in Three Kinds of Liquid Media at 37 C and 25 C

CPYG Liquid Medium at 25 C (Mycelial phase).--

The filtrates were taken from liquid CPYG medium at the end of 10, 20, and 30 days at 25 C for enzymatic activity determinations. All isolates of H. capsulatum and H. duboisii tested had relatively high activities for PNP-beta-D-glucosidase (beta-PNPGase), PNP-alpha-D-glucosidase (alpha-PNPGase), PNP-alkaline phosphatase (alk PNPPase) and PNP-acid phosphatase (acid PNPPase) for all three periods or at least for the period at 30 days. Two strains, H. capsulatum G-184 type B, and

H. duboisii were especially high for PNP alkaline phosphatase (alk PNPPase). All isolates of both species had moderately high activity of PNP-N-acetyl-beta-glucosaminase (PNPPNAGase) at 30 days. The activity for alpha and beta-PNPGase of three isolates of H. capsulatum was about the same as for H. duboisii except for "B" at the end of 10 days. There was little or no enzyme activity for PNP-alpha-D-galactoside, in straing "A" of H. capsulatum while strain "B" and H. duboisii showed activity for the former and for PNP-glucuronidase activity. The results are shown in Table 1.

CPYG Liquid Medium at 37 C (Yeast phase).--The only marked anzyme activities for the strains tested in filtrates taken from CPYG medium after 10, 20, and 30 days were alpha and beta-PNPGase in H. duboisii. This same strain also had higher alkaline and acid PNPPase. Table 2 shows the comparative enzyme activities. All activities were higher in the filtrate from the 25 C cultures except alpha and beta PNPGase from H. duboisii.

Brain Heart Infusion Broth with 0.1% Cysteine at 25 C.--Higher activity of PNPNAGase was found for 3 isolates of H. capsulatum in the filtrate for BHI broth with 0.1% cysteine at 25 C, but there was variably high activities for alpha and beta-PNPGases, and for alkaline and acid-PNPPases while alpha and beta-PNPGases of H. duboisii were consistently high. Table 3

TABLE 1.--Enzyme in the filtrate of CPYG medium after 10, 20 and 30 days at 25 C.

Histoplasma	Day	Enzyme activities (O.D./ml/hourx1000) of various substrates							
		PNP-N acetyl gluco saminide	PNP-alpha D-gluco side	PNP-beta D-gluco side	PNP-alpha D-galac toside	PNP-beta D-galac toside	PNP-glucu-ronide	PNPP-alk	PNPP-acid
capsu-latum	10	525	4350	4200	0	75	0	400	200
A	20	75	1875	2100	0	200	0	825	225
	30	700	4400	4950	25	125	400	2375	1025
	10	0	500	275	75	200	250	600	275
B	20	275	4100	4300	50	300	275	4050	175
	30	1150	4300	4350	400	300	475	4300	1375
	10	0	1550	1100	50	50	0	500	0
MSU	20	150	4450	4350	225	175	0	125	25
	30	1500	3750	4450	25	0	275	250	325
duboi-sii	10	100	3750	3750	50	0	0	3500	800
	20	0	3750	3750	0	0	0	3500	900
	30	800	4000	4100	450	250	425	4300	3800

TABLE 2.--Enzymes in the filtrate of CPYG medium after 10, 20 and 30 days at 37 C.

Histo plasma	Day	Enzyme activities (O.D./ml/hourx1000) of various substrates							
		PNP-N acetyl β gluco saminide	PNP-alpha D-gluco side	PNP-beta D-gluco side	PNP-alpha D-galac toside	PNP-beta D-galac toside	PNP-glucu-ronide	PNPP-alk	PNPP-acid
Capsu-latum	10	0	50	375	175	50	50	175	550
A	20	0	300	175	0	100	225	375	275
	30	325	625	525	150	200	25	350	800
	10	0	50	275	225	0	0	0	400
B	20	0	200	100	250	0	125	0	175
	30	325	450	425	275	75	37	37	725
	10	50	0	50	25	0	25	0	300
MSU	20	350	50	50	37	50	62	37	0
	30	325	0	125	112	75	0	287	300
	10	50	3750	3500	12	0	300	2750	1650
duboi-sii	20	0	3500	3750	150	75	150	1050	300
	30	250	4250	4250	25	75	187	350	500

TABLE 3.--Enzymes in the filtrate of BHI medium with 0.1% cysteine after 10, 20 and 30 days at 25 C.

Histo plasma	Day	Enzyme activities (O.D./ml/hourx1000) of various substrates							
		PNP-N acetyl gluco saminide	PNP-alpha D-gluco side	PNP-beta D-gluco side	PNP-alpha D-galac toside	PNP-beta D-galac toside	PNP-glucu-ronide	PNPP-alk	PNPP-acid
Capsu-latum	10	600	525	400	400	75	250	2250	775
A	20	525	1025	700	225	275	200	2750	1800
	30	3250	2500	4050	200	275	300	1175	775
	10	450	900	600	250	450	250	3500	2500
B	20	550	2750	3250	400	375	675	4100	2500
	30	1700	3500	4300	350	550	325	3000	1400
	10	250	700	875	100	350	325	3000	675
MSU	20	625	1150	950	200	150	250	3750	3500
	30	875	2000	1975	175	300	275	1750	900
	10	550	3750	3500	475	400	350	3750	2250
duboi-sii	20	275	3750	3500	275	200	250	840	3000
	30	725	4350	3750	250	350	275	1875	1100

illustrates these results. Most reactions were higher in the CYPG agar except for two strains of "A" and "B".

Brain Heart Infusion Broth with 0.1% Cysteine at 37 C.--Histoplasma duboisii had higher activities in alpha and beta-PNPGase and alkaline-PNPPase than the isolates of H. capsulatum when grown in BHI with 0.1% cysteine at 37 C. The results were similar to those in CPYG medium at 37 C but lower in the activities for alpha and beta-PNPGase and higher for alkaline-PNPPase. All isolates of H. capsulatum had low activity for every enzyme tested. Table 4 summarizes the results.

Sabouraud Dextrose Broth at 25 C.--All isolates had more or relatively low activities for all enxymes tested until the end of the 30 days incubation in Sabouraud dextrose broth at 25 C. Apparently the activity for alpha and beta-PNPGase of all isolates increased at the end of the 30 day incubation period being much higher for H. duboisii than for strains of H. capsulatum. Alkaline and acid-PNPPase activities of all strains except H. capsulatum G-184 type "A" rose at 30 days. Table 5 shows the results.

Some Characterisitcs of PNP-Alpha and Beta Glucosidase

Enzyme Production in Relation to Age of Culture in Liquid Medium.--In 100 ml CPYG medium at 37 C with 2 ml of inoculum containing approximately 829 x 10^6 cells per ml, the total cell counts of H. duboisii increased

TABLE 4.--Enzymes in the filtrate of BHI medium with 0.1% cysteine after 10, 20 and 30 days at 37 C.

Histo plasma	Day	Enzyme activities (O.D./ml/hourx1000) of various substrates							
		PNP-N acetyl gluco saminide	PNP-alpha D-gluco side	PNP-beta D-gluco side	PNP-alpha D-galac toside	PNP-beta D-galac toside	PNP-glucu-ronide	PNPP-alk	PNPP-acid
Capsu-latum	10	0	0	0	550	0	--	875	550
A	20	0	125	100	--	75	--	525	275
	30	225	450	75	--	0	--	100	325
	10	200	0	0	--	--	--	300	100
B	20	175	125	0	225	50	75	350	100
	30	0	350	625	100	50	300	150	175
	10	0	0	0	250	175	0	75	50
MSU	20	550	250	100	300	325	0	350	0
	30	150	0	150	50	50	0	325	225
	10	200	250	250	150	350	0	2375	375
duboi-sii	20	100	500	525	150	325	--	3000	875
	30	150	3750	3000	100	275	--	0	250

TABLE 5.--Enzyme in the filtrate of Sabouraud medium after 10, 20 and 30 days at 25 C.

Histo plasma	Day	Enzyme activities (O.D./ml/hourx1000) of various substrates							
		PNP-N acetyl gluco saminide	PNP-alpha D-gluco side	PNP-beta D-gluco side	PNP-alpha D-galac toside	PNP-beta D-galac toside	PNP-glucu-ronide	PNPP-alk	PNPP-acid
Capsu-latum	10	100	0	0	0	0	0	0	0
A	20	250	150	0	0	125	75	100	125
	30	225	225	300	0	125	25	200	125
	10	0	0	100	125	100	0	0	100
B	20	100	0	0	175	100	0	0	0
	30	300	500	675	275	125	175	725	425
	10	0	0	0	25	0	0	0	0
MSU	20	125	50	0	175	125	0	150	0
	30	0	300	650	0	0	25	325	300
	10	100	0	0	125	0	75	0	25
duboi-sii	20	300	425	0	175	50	125	500	50
	30	200	1725	1850	375	100	150	350	375

rapidly during the third to the fifth day of incubation. The peak of growth occurred on the seventh day. The production of alpha and beta-PNPGase started to rise rapidly on the ninth day or 2 days after the growth peak and then, continued to increase until the end of the 30 days incubation. The total cell count for the organism decreased during the last 10 days of incubation. Table 6 and Figure 8 show the results. In comparison, H. capsulatum strain G-184 "A" and "B" had low alpha and beta-PNPGase activities, never higher than 150 units (0.D./ml.hour x 1000) at any time in contrast to over 11,000 units for H. duboisii at the end of the 30 day period.

Enzyme Production in Relation to the Age of Culture on Solid Medium.--The production of alpha and beta-PNPGases were detected in the culture medium (CPYG agar plate) by the plug method after 4 day incubation at 37 C. The activities of these enzymes kept rising in H. duboisii throughout the 30 day period. Relatively low activities of these two enzymes were found in the culture medium with H. capsulatum, strain G-184, type "A" and "B" and MSU strain. The data are shown in Table 7.

TABLE 6.--Alpha and beta-PNPGase production in the filtrate during growth of H. duboisii in CPYG medium at 37 C.

No. of day Incubation	Enzyme activities (O.D./ml/hourx1000)		Total Cell count$x4x10^6$/ml
	alpha-PNPGase	beta-PNPGase	
1	0	0	3.36
2	0	0	7.43
3	0	0	19.90
4	0	0	56.81
5	0	0	137.34
6	0	0	186.45
7	100	35	224.84
8	75	75	197.81
9	2550	2200	217.25
11	7500	7850	200.62
22	10950	11100	190.62
25	9750	9650	178.12
30	11600	11000	171.35

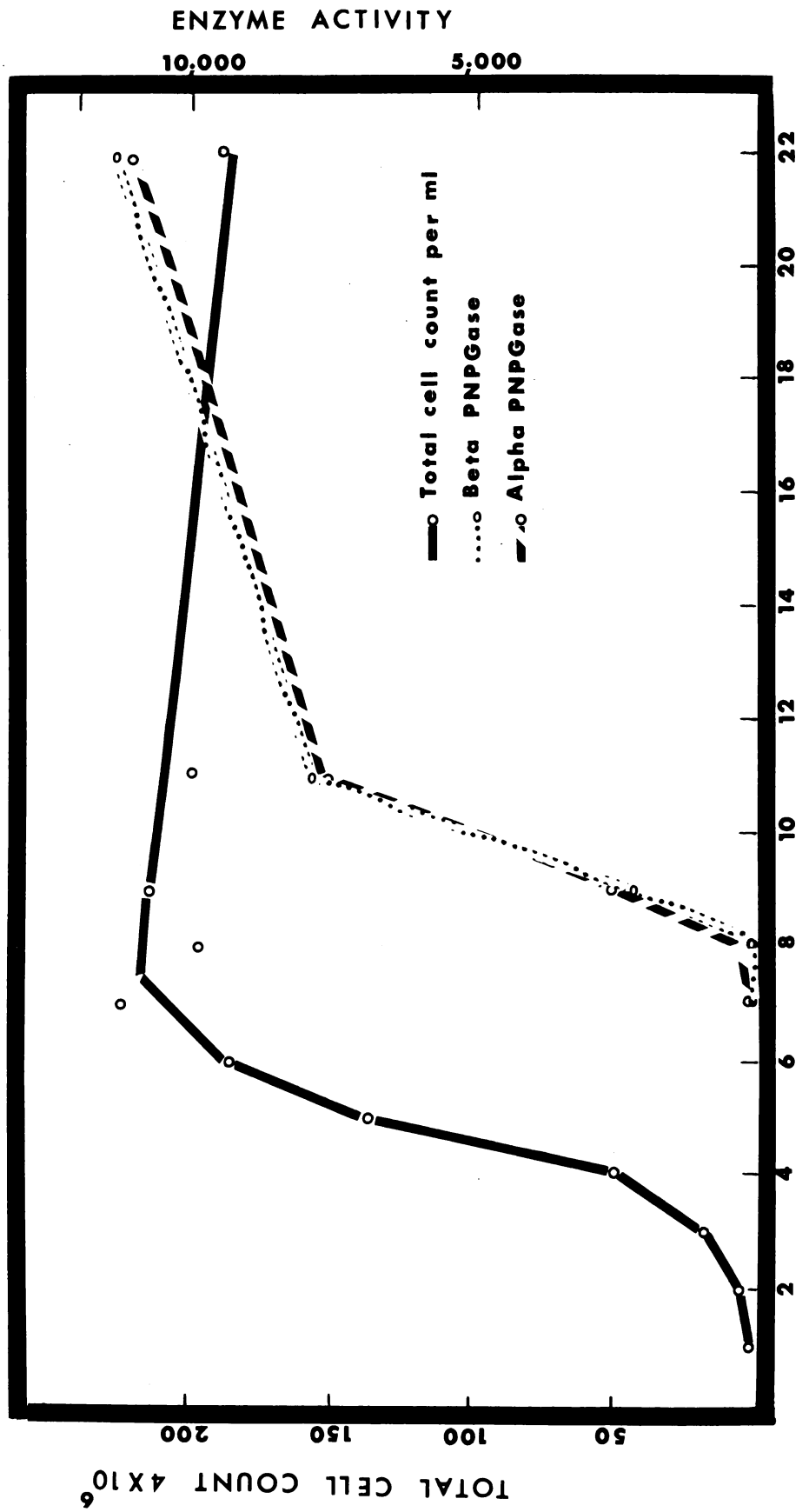


FIGURE 8.--Growth curve and enzyme productions of Histoplasma duboisii in CPYG medium at 37 C.

TABLE 7.--Enzyme activities of alpha and beta-PNPGases produced by H. capsulatum and H. duboisii in the CPYG agar plates over a 30 day period at 37 C.

Organism	Activity (O.D./agar plug/hour x 1000)																	
	2 days		4 days		6 days		8 days		11 days		14 days		20 days		26 days		30 days	
	1*	2*	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
H. capsulatum G-184 A	0	0	0	0	0	0	0	0	0	0	60	30	10	20	10	20	10	20
G-184 B	10	20	10	10	0	0	0	20	80	70	80	10	20	20	20	20	20	20
MSU	30	20	10	20	0	0	0	0	0	10	10	0	0	20	0	20	0	20
H. duboisii	40	20	90	80	120	130	200	210	290	380	400	430	640	660	860	750	960	850

*1 PNP-alpha-D-glucoside; *2 PNP-beta-D-glucoside.

Optimal Temperature and pH.--At 4 C alpha and beta-PNPGases were low in activity in the liquid filtrate of CPYG medium for H. duboisii MSU strain grown at 37 C. An increase in the incubation temperature increased the activity of both enzymes. At 69 C, both enzymes showed the highest activity for the temperatures selected. Table 8 and Figure 9 show the results.

The alpha and beta PNPGases in the filtrate lost their activities after 3 minutes in a steamer, or after one hour at 70 C. The optimal pH for alpha-PNPGase in H. duboisii was found to be pH 4.6 and pH 4.8 for beta-PNPGase. A rapid drop occurs in activity of these enzymes above or below these points. Table 9 and Figure 10 illustrate the effects of pH changes.

Effect of Storage at -20 C.--The alpha and beta-PNPGases in the filtrate of H. duboisii MSU strain in CPYG medium after storage in the freezer for 90 days showed no deterioration in enzyme activity.

Kinetic Plots of Alpha and Beta-PNPGases.--Both enzymes produced a linear increase in activity up to 90 minutes then leveled off. Figure 11 shows the kinetics of both enzymes, Table 10 lists the data of this experiment.

Inhibition Test.--Glucose, mannose, galactose and d-levulose were tested for inhibition effects on alpha and beta-PNPGases. Greater inhibition of alpha and beta-PNPGase occurred at 0.46 M than 0.04 M for glucose and mannose. No inhibition was evident

TABLE 8.--Effect of temperature on activity of alpha and beta-PNPGases produced by H. duboisii MSU strain.

Temperature 1 hour	Activity (O.D./ml/hour x 1000)	
	alpha-PNPGase	beta-PNPGase
4 C	200	750
25 C	2700	2100
37 C	11700	11200
59 C	55200	54000
69 C	120000	117300
75 C	12700	11800

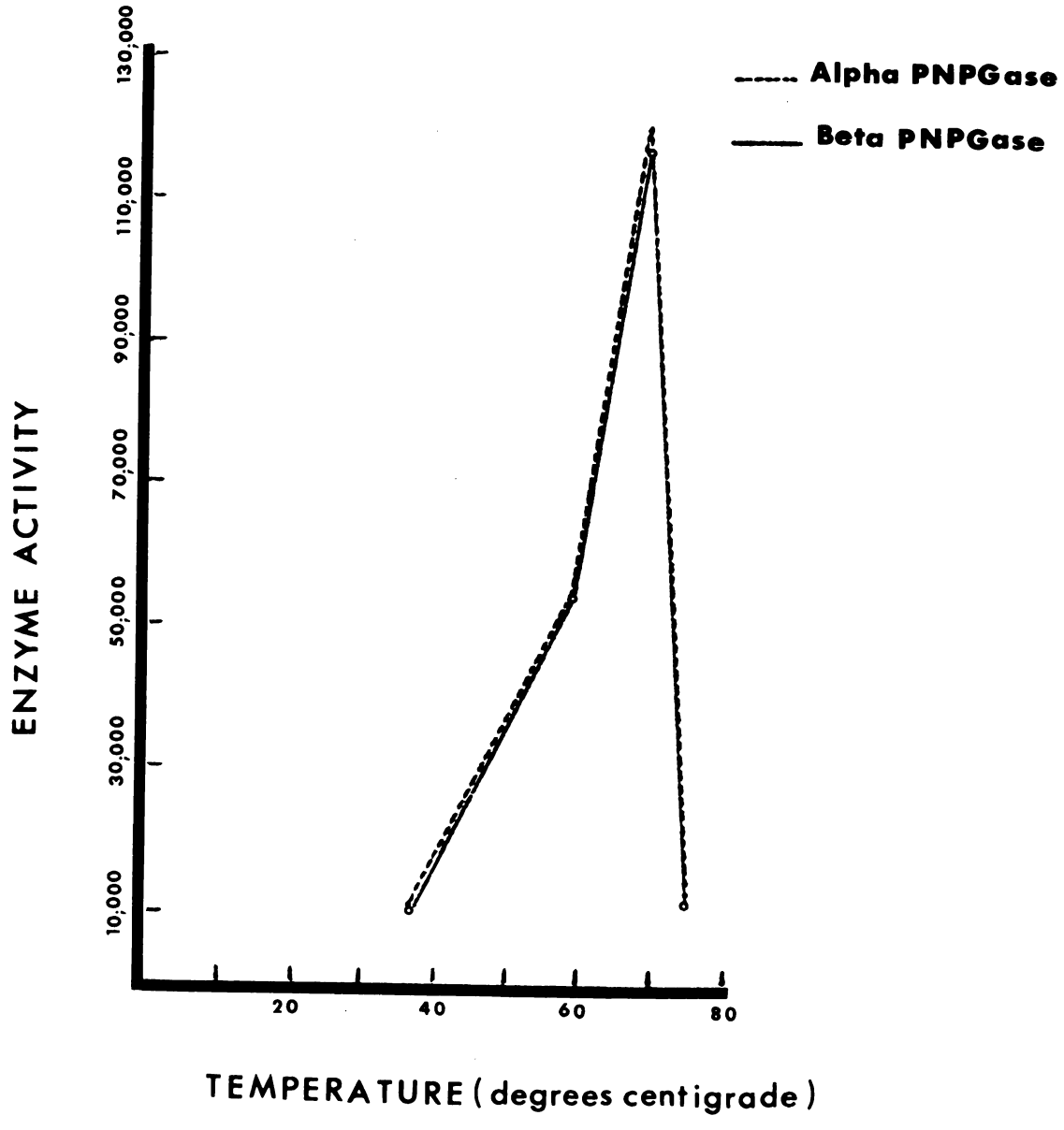


FIGURE 9.--Effects of temperature on alpha and beta-PNPGases from the culture filtrate of H. duboisii at 37 C.

TABLE 9.--Effect of pH on activity of alpha and beta-PNPGases from the filtrate from H. duboisii MSU strain.

pH	activity (O.D./ml/hour x 1000)	
	alpha-PNPGase	beta-PNPGase
3.0	9400	8600
4.0	12000	11000
4.2	11000	11000
4.4	12000	11100
4.6	12500	11100
4.8	12000	11800
5.0	12000	11100
5.3	11800	11100
5.0	12000	11000
6.0	11000	8600
7.0	7500	7200
8.0	4900	3800
9.0	2500	2400

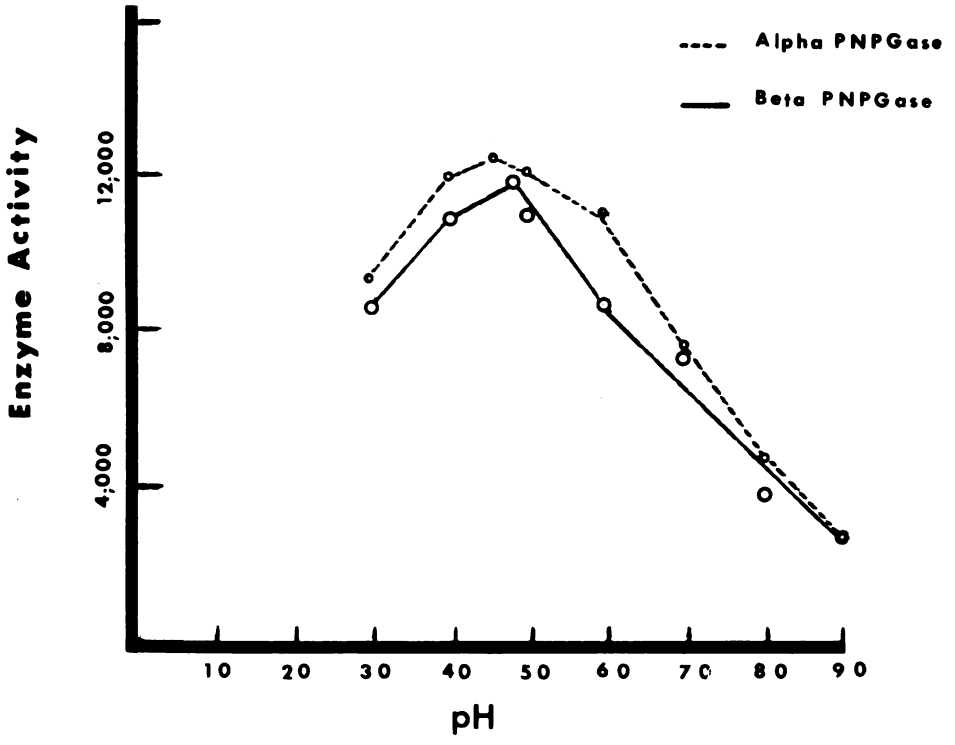


FIGURE 10.--Effects of pH on alpha and beta-PNPGases from the culture filtrate of H. duboisii at 37 C.

TABLE 10.--Kinetics of alpha and beta-PNPGases for H. duboisii MSU strain at 37 C.

Time (minute)	Activity (O.D./ml/hour x 1000)	
	alpha-PNPGase	beta-PNPGase
0	0	0
5	0	600
10	600	1300
15	1500	1950
25	3200	3500
40	5400	5400
60	8400	7400
90	10000	9000
212	11000	9500

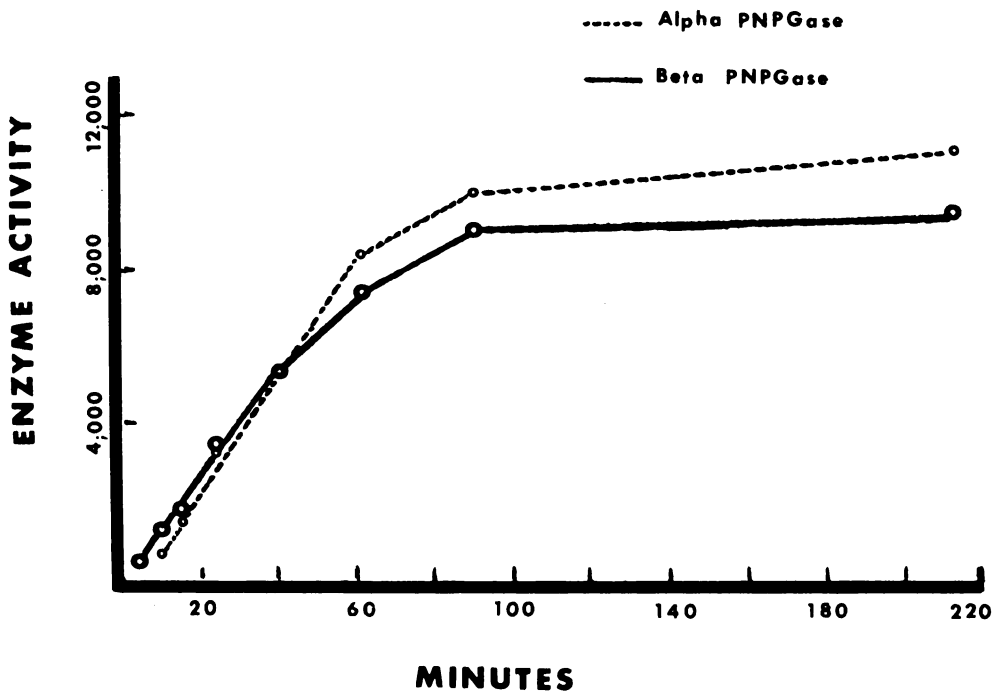


FIGURE 11.--Kinetics of alpha and beta-PNPGase activities in the culture filtrate of H. duboisii in CPYG medium at 37 C.

when d-levulose and galactose were used at a concentration of 0.46 M. Figure 12 and Table 11 summarizes the results .

Effects on Enzyme Production due to the Modifications in Liquid CPYG Medium.--

a. Casein and the amounts of glucose

The amount of glucose in the medium had an important effect on the production of alpha and beta-PNPGases by H. duboisii MSU strain at 37 C. It was found that 1.5% glucose in CPYG liquid medium was the best for the highest activities of both enzymes. Vitamin free casein (Difco) was one of the essential factors for enzyme production in this medium and could not be replaced by casamino acids, an acid hydrolysis product of casein. Some essential factor(s) in the casein for the two enzyme productions is suspected to be lost during acid hydrolysis of casein. Attempts to find this factor(s) by adding several salts and amino acids in casamino acids to check whether they could induce enzyme production were not successful. The results are shown in Table 12.

TABLE 11.--Inhibition effects of glucose and mannose on alpha and beta-PNPGases from the culture filtrate of H. duboisii MSU strain in CPYG medium at 37 C.

Inhibitor		Activity (O.D./ml/hour x 1000)			
Sugar	Conc.	alpha-PNPGase	%inhibition	beta-PNPGase	%inhibition
No inhibition (water)		17600	0	17800	0
Glucose	0.46M	1040	94.10	1080	93.94
Glucose	0.09M	2900	83.53	3500	80.34
Glucose	0.04M	4400	75.00	6600	62.93
Mannose	0.46M	8300	52.85	9900	44.38
Mannose	0.09M	1360	22.73	15100	15.17
Mannose	0.04M	14800	15.91	16700	6.20
Galactose	0.46M	17600	0	17800	0
d-Levulose	0.46M	17600	0	17800	0

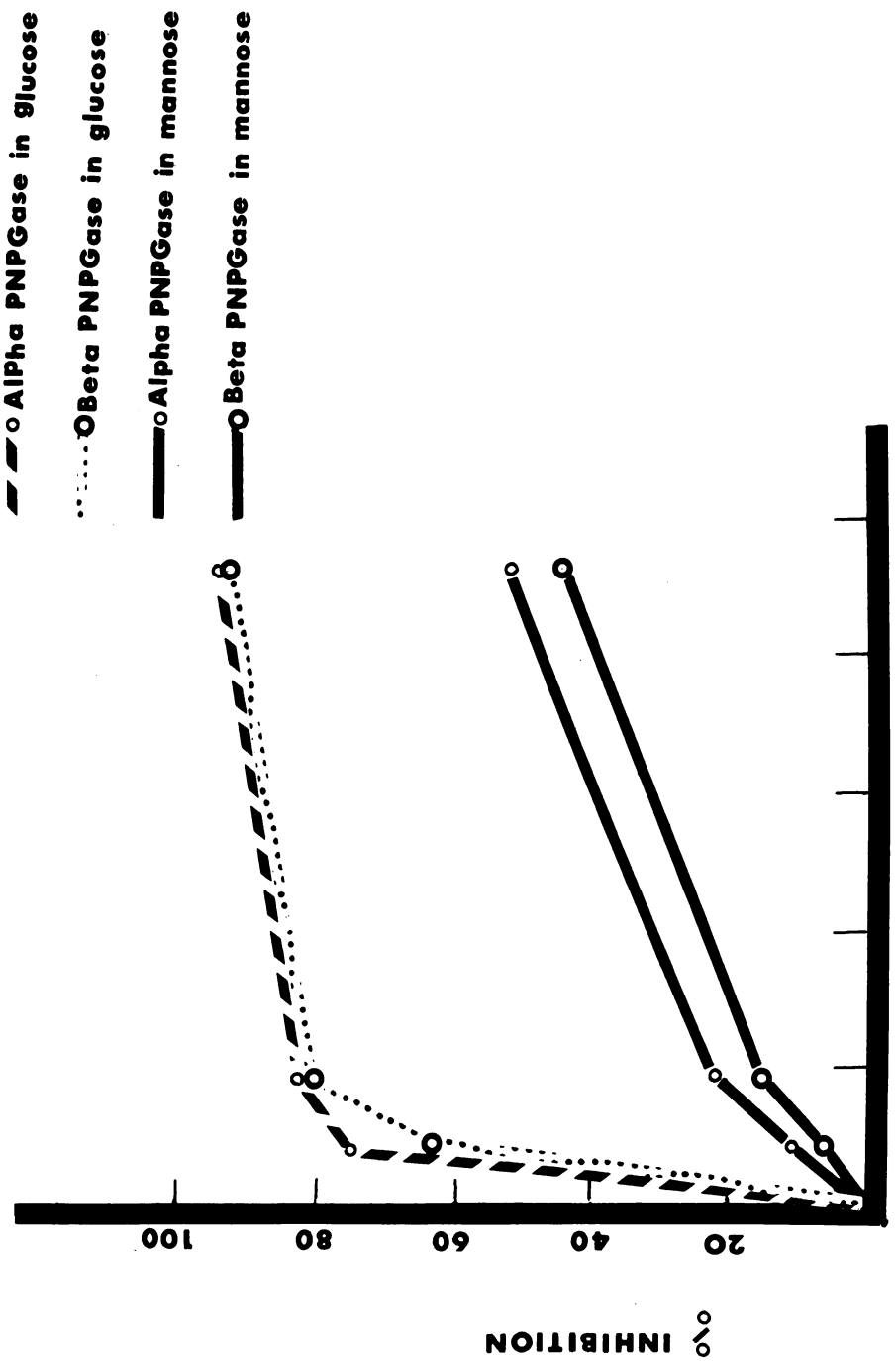


FIGURE 12.--Inhibition effects of glucose and mannose on alpha and beta-PNPGase from the culture filtrate of H. duboisii in CPYG medium at 37 C.

TABLE 12.--Effects of casein and amounts of glucose on enzyme production by H. duboisii in CPYG liquid medium in 15 days at 37 C.

Modifications of medium	Activity (O.D./ml/hour x 1000)	
	alpha-PNPGase	beta-PNPGase
Glucose 0.0%	1800	1500
Glucose 0.5%	6100	4900
Glucose 1.0%	8400	7300
Glucose 1.5%	21000	18000
Glucose 2.0%	17800	16800
Casamino acids replaced casein	0	0
Casamino acids with lmg/ml dextrin	0	0
Cas. acids with lmg/ml ferric phosphate	370	70
Cas. acids with lmg/ml ferric citrate	150	100
Cas. acids with lmg/ml sodium phosphate	250	100
Cas. acids with lmg/ml tryptophan	600	400
Cas. acids with lmg/ml serine	300	200

The same CPYG liquid medium in which 1.5% agar (Difco) was added and the agar medium near the edge of the colony was removed as agar blocks and assayed for alpha and beta-PNPGases. Lower but somewhat similar results occurred for effects on the amounts of glucose in the medium, with 1.5% best for enzyme production. Casamino acids could be substituted for casein as alpha and beta-PNPGase activities were still found in the agar medium as shown in Table 13.

b. Sugars

Comparatively, the production of alpha and beta-PNPGase activities were higher in cellobiose than in lactose, soluble starch, and dextrin. Cellobiose was nearly as good for alpha and beta-PNPgase activity but glucose was still better as shown in Table 14.

Identification of alpha and beta-PNPGase Fractions by Disc Electrophoresis of Lyophilized Culture Filtrates from H. duboisii in CPYG Liquid Medium at 37 C.--Enzyme fractions in the lyophilized culture filtrate gels reacted with the substrates (PNP-alpha-D-glucoside and PNP-beta-D-glucoside) which diffused into the gels resulting in the liberation of free paranitrophenol at the sites of alpha and beta-PNPGases in the gels. When

TABLE 13.--Effects of the amounts of glucose and casein in solid CPYG medium on enzyme production by H. duboisii in 10 days at 37 C.

Modifications of medium	Activity (O.D./agar plug/hour x 1000)	
	alpha-PNPGase	beta-PNPGase
Glucose 0%	360	310
Glucose 0.5%	390	330
Glucose 1.0%	450	600
Glucose 1.5%	930	750
Glucose 2.0%	570	690
Casamino acids replaced casein (glucose 1.5%)	310	250

TABLE 14.--Effects of different sugars and carbohydrates in liquid CPYG medium on enzyme productions by H. duboisii in 15 days at 37 C.

Modifications of medium Carbon Source		Activity (O.D./ml/hour x 1000) alpha-PNPGase	Activity (O.D./ml/hour x 1000) beta-PNPGase	Total cell count
CPY minus Glucose	0.0%	460	260	4.29
D-cellobiose	1.0%	18400	17600	180.20
D-lactose	1.0%	4390	3950	48.80
Soluble starch	1.0%	3200	2900	49.00
Dextrin	1.0%	2900	2680	59.40
Cellobiose*	1.0%	9600	9400	127.80
Glucose*	1.0%	11000	14000	151.20

*The inoculum was two mls of a cell suspension of 60.4 x 10^6 cells/ml of 4 day old stock culture.

the gels were put into 1.0 M Tris, paranitrophenol developed a yellow color band in the gels at the site(s) of the enzymes.

The gel which was put into PNP-alpha-D-glucoside developed one yellow band at the rf 40.0. When the gel was put into PNP-beta-D-glucoside, one yellow band developed at rf 40.0. The yellow band of free paranitrophenol in each gel corresponded with the stained protein band of the third gel of the triplicate. Figure 13a and 13b show the diagram and the photograph of the bands.

Alpha and beta-PNPGases in other isolates of H. capsulatum and H. duboisii on Sabouraud Dextrose Agar for 30 days at 25 C.--All 12 isolates of H. capsulatum and H. duboisii were cultured on Sabourand dextrose agar for 30 days at 25 C. Agar discs were used for assay. Two isolates of H. duboisii gave higher activities for both alpha and beta-PNPGases than the 10 isolates of H. capsulatum. One (B-937) of the two isolates of H. duboisii not previously tested for alpha and beta-PNPGases gave a slightly higher activity while the other (B-939) was significantly higher. The results are shown in Table 15.

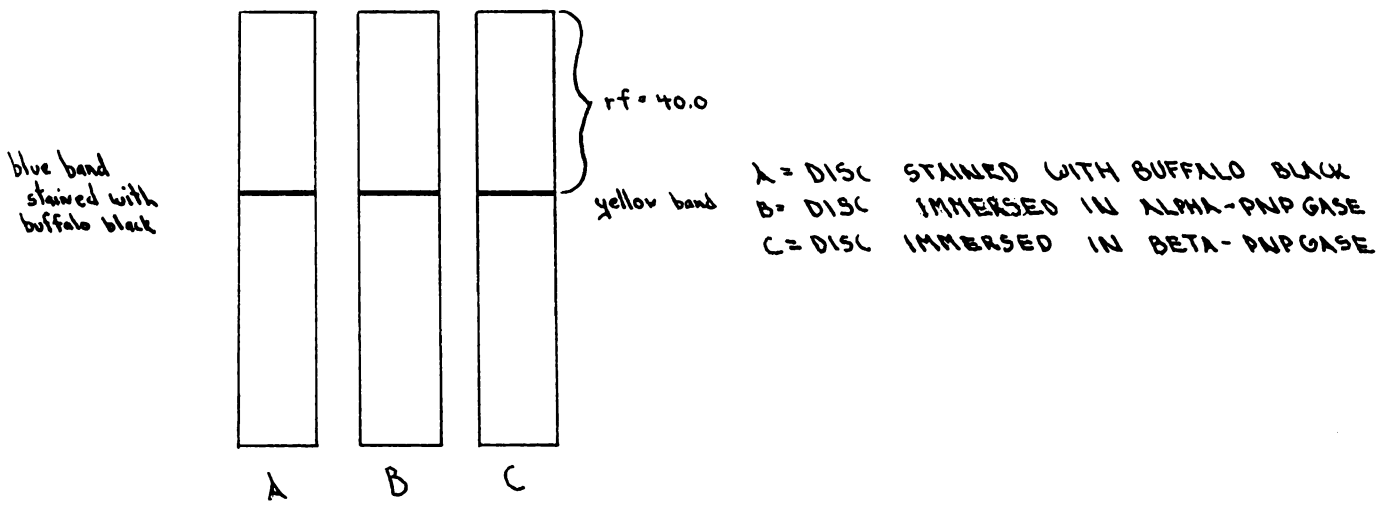


FIGURE 13a.--Diagram of electrophoretic patterns in acrylamide gels of the lyophilized filtrate from H. duboisii in CPYG for 30 days at 37 C compared with the band produced by alpha and beta-PNPGase activities.

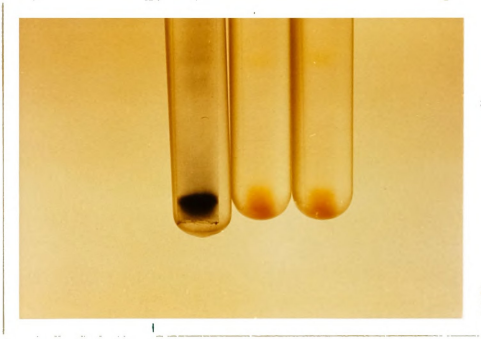


FIGURE 13b.--A photograph of electrophoretic patterns in acrylamide gels of the lyophilized filtrate from H. duboisii in CPYG medium for 30 days at 37 C, compared with the band produced by alpha and beta-PNPGases.

TABLE 15.--The production of alpha and beta-PNPGases in Sabouraud dextrose agar of additional strains of H. capsulatum and H. duboisii for 30 days incubation at 25 C.

Organism	Activity (O.D./agar plug/hour x 1000)	
	alpha-PNPGase	beta-PNPGase
H. capsulatum B-875	130	130
H. capsulatum B-293	10	40
H. capsulatum B-621	30	100
H. capsulatum G-186 type A	10	40
H. capsulatum G-186 type B	40	30
H. capsulatum 219 type A	160	100
H. capsulatum 219 type B	30	70
H. capsulatum 488	60	60
H. capsulatum D-213	180	170
H. capsulatum 68-46	70	70
H. duboisii B-937	200	200
H. duboisii B-939	370	280

The Distribution of PNP-alkaline Phosphatase in Polyacrylamide Gels from Disc Electrophoresis of Lyophilized Culture Filtrates of Strains of H. capsulatum and H. duboisii in CPYG Broth for 30 days at 25 C

Three strains of H. capsulatum 184 G type B, 186 G type B, and 219 type B, and three strains of H. duboisii MSU strain, B 937, and B 939 were fractionsted by disc electrophoresis, from lyophilized culture filtrates after growing in CPYG broth at 25 C. The activity of PNP-alkaline phosphatase produced by 3 strains of H. capsulatum and 3 strains of H. duboisii was variable. Histoplasma capsulatum strain G-186 type "B" and H. duboisii strain B-937 had high activities while H. capsulatum strain 222 type "B" produced low acitivity for alkaline-PNPPase. Significantly, all strains of both species had the peak of alkaline-PNPPase activities in the gel section number 4, or in between 1.5 - 2.0 cm from the starting point of electrophoresis in the small pore gels. Table 16 and Figure 14 show these results.

A Study of Alpha and Beta-PNPGases and Alkaline-PNPPase Produced by H. capsulatum Type "A" and "B" When Grown on BHI Agar Plates with 0.1% Cysteine for 21 Days at 25 C

Three strains of H. capsulatum type "A" and "B" when grown on BHI agar with 0.1% cysteine at 25 C for 20 days were checked for enzyme activity by the agar

TABLE 16.--The distribution of alkaline-PNPPase in polyacrylamide gels from disc electrophoresis of lyophilize culture filtrates of 3 strains of H. capsulatum and H. duboisii in CPYG media for 30 days at 25 C.

Organism	Activity* of alk-PNPPase in section number**							
	1	2	3	4	5	6	7	8
H. capsulatum G-184B	70	170	450	490	200	20	20	10
H. capsulatum 222B	10	25	30	75	45	15	15	10
H. capsulatum G-186B	40	65	180	1400	1050	115	20	15
H. duboisii MSU	65	140	310	780	250	15	5	5
H. duboisii B-937	20	150	500	1400	1100	140	10	10
H. duboisii B-939	20	35	250	850	510	50	10	10

*O.D./gel section/hour x 1000

**Each section = 5mm.

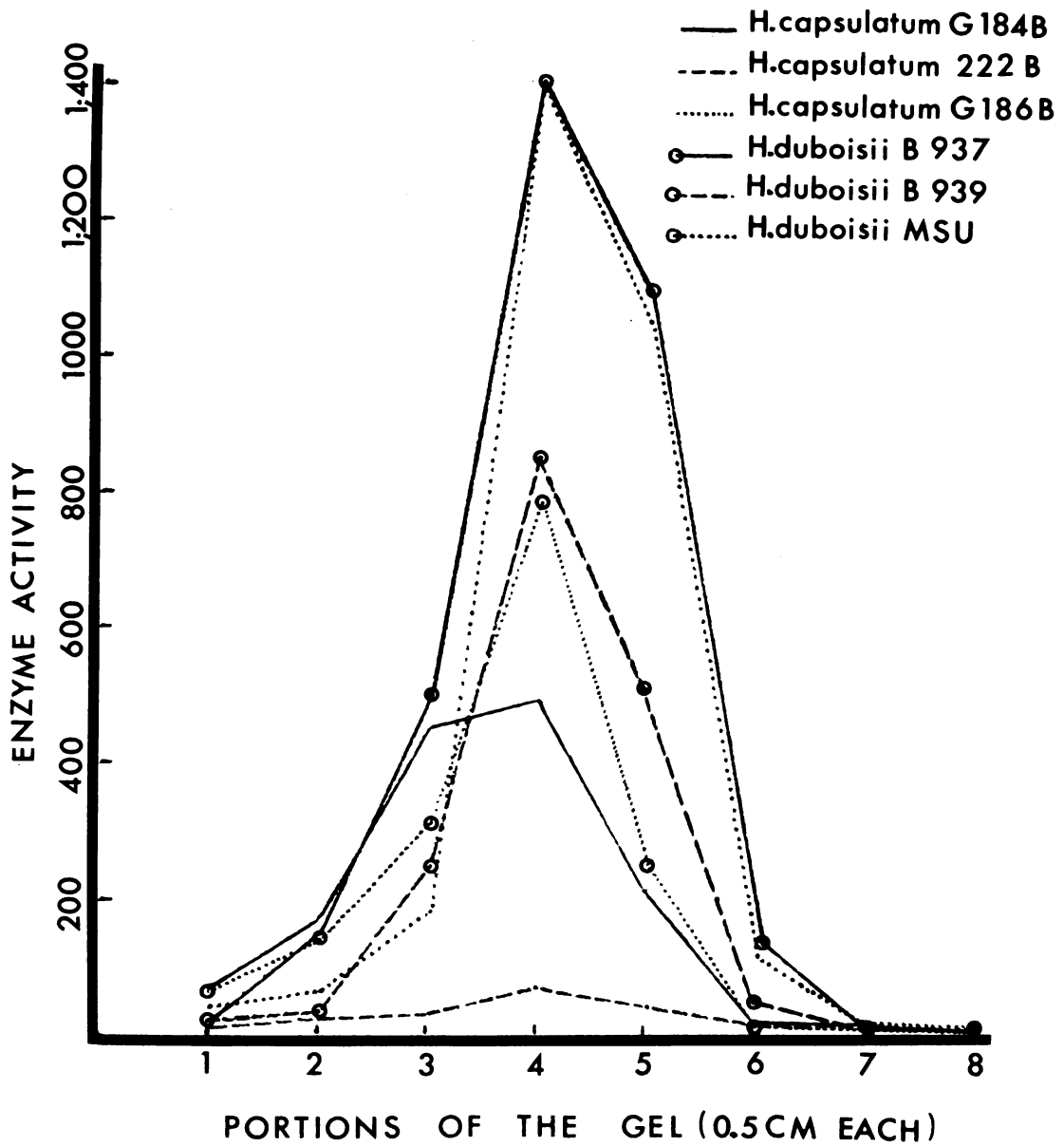


FIGURE 14.--Graphic diagram showing the distribution of alk-PNPPase in polyacrylamide gels.

plug method. Both types of H. capsulatum produced high activity of alpha and beta-PNPGases and low activity of alk-PNPPase. Significantly, type "A" had 2-8 time higher activity than type "B" for alpha and beta-PNPGases and 2-5 time higher activity than type "B" for alk-PNPPase. The results are listed in Table 17.

Study of Alpha and Beta-PNPGase and Alkaline-PNPPase Activities in 5 Strains of H. capsulatum and H. duboisii in CPYG Agar Plates After 9 Days at 37 C

High activity of alkaline-PNPPase was found in the plugs taken from near the colonies in CPYG agar medium of cultures at 37 C of 5 strains of H. duboisii while 5 strains of H. capsulatum produced low activity of the same enzyme. Histoplasma capsulatum strain B 913, B 329, 105 and H. duboisii strain B 650, B 651, B 652 were received from Dr. L. Georg, Communication Disease Center, Atlanta, Ga. All six isolates have the same serotype based on fluorescent antibody technique (Kaufman and Blumer, 1961). Histoplasma duboisii MSU strain and H. capsulatum 222 A were the only ones which produced high activities of alpha and beta-PNPGase. The data are shown in Table 18.

TABLE 17.--Comparison of alpha and beta-PNPGases and alkaline-PNPPase activities produced by 3 strains of H. capsulatum type "A" and "B" on BHI agar plates with 0.1% cysteine for 21 days at 25 C.

Organism	Enzyme activity (O.D./agar plug/hour x 1000)			
	alpha-PNPGase	beta-PNPGase	alk-PNPPase	colo. dia. (cm)
H. capsulatum 219 type A	2640	2360	80	2.8
H. capsulatum 219 type B	1080	1240	27	3.2
H. capsulatum 222 type A	2200	840	60	3.5
H. capsulatum 222 type B	320	380	12	3.0
H. capsulatum G186 type A	2040	1840	80	2.0
H. capsulatum G186 type B	280	300	35	3.5

TABLE 18.--Activities of alpha and beta-PNPGases and alkaline PNPPase of strains of H. capsulatum and H. duboisii on CPYG agar plates for 9 days at 37 C.

Organism	Enzyme activity (O.D./agar plug/hour x 1000)		
	alpha-PNPGase	beta-PNPGase	alk-PNPPase
H. capsulatum B 913	5	10	45
H. capsulatum B 329	0	0	40
H. capsulatum 105	5	5	65
H. capsulatum MSU	20	10	60
H. capsulatum 222 A	170	130	75
H. duboisii B 650	5	10	330
H. duboisii B 651	0	0	560
H. duboisii B 652	5	0	375
H. duboisii B 939	0	0	340
H. duboisii MSU	290	250	185

Fine Structure in Young Hyphae of Histoplasma duboisii and Histoplasma capsulatum

The mycelial phase of H. duboisii grown for 10 days in Sabouraud dextrose broth (Figure 15) showed fine structure resembling H. capsulatum. It is multinucleate (N) in a hyphal cell with prominent nucleoli (NCL). A small intracytoplasmic membrane system (IM) was found close to the plasma membrane (PM) (Figure 16).

In the limited number of sections of H. duboisii observed, Woronin bodies were not found associated in the area of the septum (Figure 17). On the other hand, a section of a hypha from H. capsulatum showed two Woronin body-like structures (Figure 18) in the area near the septum similar to those reported by Garrison (1970).

Attempts to Develop a Differential Medium for Histoplasma capsulatum and H. duboisii

Yeast Phase

The MSU strain of H. duboisii began to show a color change from cream color to purple after 10 days at 37 C on CPYG agar containing either the combination of ferrous sulfate and sodium thiosulfate or ferrous sulfate alone in all concentrations tested. The color did not change in the colonies growing on the CPYG medium that contained ferrous sulfate with the addition of cysteine for up to 30 days at 37 C. The colonies of all five strains of H. capsulatum remained cream color until the end of 30 days at 37 C. The photograph

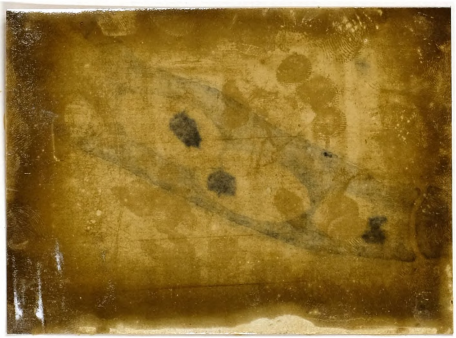


FIGURE 15.--A segment of a hyphal cell of Histoplasma duboisii showing three nuclei x 18,000.

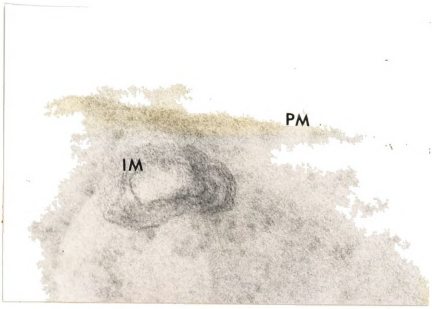


FIGURE 16.--A segment of a hyphal cell of Histoplasma duboisii showing intracytoplasmic membrane system (IM) near the plasma membrane (PM) x 102,000.

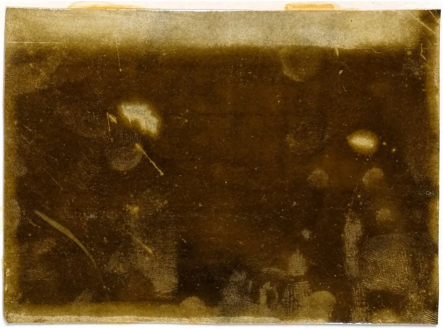


FIGURE 17.--A septum in a segment of a hypha of H. duboisii, Woronin bodies were not found x 57,000.

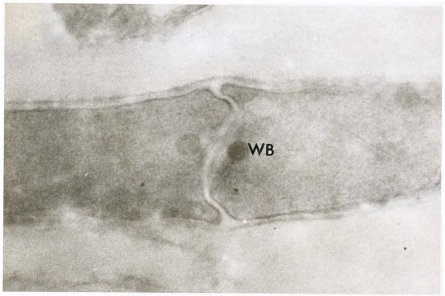


FIGURE 18.--A segment of a hyphal cell of Histoplasma capsulatum showing a septum with two Woronin body-like structures (WB) x 54,000.

showing the difference in color of the colonies of these two organisms is illustrated in Figure 19. However, when additional strains of H. duboisii strains were grown on this modified medium, the colonies failed to change to the purple color characteristic of H. duboisii MSU strain. When the mycelial phase of both H. capsulatum and H. duboisii were used as inoculum in this medium and incubated at 37 C, the colonies became purple after a 2 or 3 subcultures on this medium.

Mycelial Phase

An indicator medium with phenol red was prepared to investigate any changes in the pH in the media during the growth of H. capsulatum strains 488, B-621, B-875, and B-293 and H. duboisii strains B-937, B-938, B-939, and MSU. All cultures inoculated with the mycelial phase were grown in slants at 25 C. The color change of the medium from yellow to red was noted during colony development. More strains of H. capsulatum turned the color of the medium red than H. duboisii. Histoplasma capsulatum strains produced alkaline reactions, as indicated by the medium turning red, in variable lengths of time. After 9 days, all four strains of H. capsulatum changed the color in the medium red while the medium for 3 strains of H. duboisii

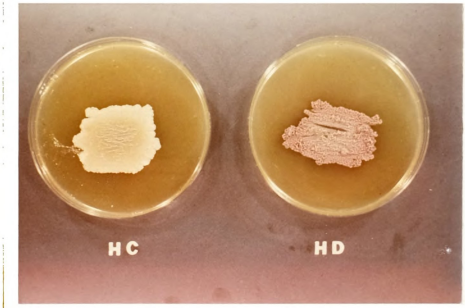


FIGURE 19.--A photograph of a purple colony of H. duboisii MSU strain and a colony of H. capsulatum strain G-184A on a modified medium for 18 days at 37 C.

(B 937, B 938, B 939) was still yellow. Only the MSU strain showed moderate degree of alkaline reaction. In order to make this medium a better one for the differentiation of these two species, the concentration of the yeast extract in the medium was adjusted to vary from 0.5 g/l to 1.5 g/l. Histoplasma capsulatum strains 488, B-293, B-621, B-875, B-913, 105 and B-329 and H. duboisii strains B-937, B-938, B-939, B-650, B-651, B-652 and MSU were grown on this medium. The best results were obtained on the medium containing 1.5 g/l yeast extract for 12 days at 25 C. All strains of H. capsulatum changed the color in the medium red while only 2 of the 7 strains of H. duboisii (B-652, MSU) showed some red color.

DISCUSSION

Disc Electrophoresis of Soluble Protein in Culture Filtrate

The method followed for determining the protein fractions of Histoplasma capsulatum and H. duboisii was similar to that used by Schechter et al. (1966) in his investigations of the electrophoretic patterns of dermatophytes and Coccidioides immitis. In preliminary experiments, the selection of an appropriate medium was necessary so that the organisms would produce proteins in the filtrate which would yield more distinct electrophoretic patterns in polyacrylamide gels. Different liquid media including brain heart infusion broth plus 0.1% cysteine, Sabouraud dextrose broth, and CPYG medium (Beneke, et al.,1969) were used. It was found that the culture filtrate of Sabouraud dextrose broth and brain heart infusion broth with 0.1% cysteine at 25 C and 37 C did not show good electrophoretic patterns even though these filtrates had been concentrated by either saturated ammonium sulphate or by lyophilization. The lyophilized culture filtrates of both phases of the organisms in CPYG medium produced the best results by disc electrophoresis. Therefore CPYG medium was selected for studying the electrophretic patterns of various strains of H. capsulatum and H. Duboisii.

The CPYG medium is highly enriched with amino acids in the casein, vitamin B complex in yeast extract, nitrogen in the neopeptone (Difco), and carbon in the 1% glucose. Thus, the medium will meet the nutritional requirements of H. capsulatum which is know to require certain vitamins (Pine, 1957; Salvin, 1949) and different amino acids (Rowley and Pine, 1955a; Pine, 1955b) for growth. In such an enrichment medium as CYPG, the organisms have sufficient nutrients to synthesize proteins, which resulted in more fractions in disc electrophoresis. In later experiments it was found that acetone precipitation of proteins was superior to lyophilization of the culture filtrate of Histoplasma species for concentration of proteins used in disc electrophoresis. On this basis, the acetone precipitation method was employed for the later experiments. Since the inocula were standardized, one would expect every subculture of the same strain to grow at the same rate and to produce soluble proteins at the same stage of growth, and to give the same uniform electrophoretic patterns. A strain of H. duboisii (MSU) was used to determine the effect of the amount of inoculum on protein fractions by disc electrophoresis. An increase in the amount of inoculum from 1800×10^6 to 3600×10^6 cells per ml or 100% showed no difference in the electrophoretic patterns after 30 days incubation. For this reason the

amount of inoculum was not critical if the amount was sufficient and the incubation period was long enough. Although Shechter et al. (1966), in their experiment with dermatophytes to determine phylogenetic relationship by disc electrophoresis, did not standardize the inocula, comparable results were obtained. Although the growth curves of various strains of H. capsulatum and H. duboisii in CPYG medium were not determined, the electrophoretic patterns of soluble proteins in the culture filtrates at 30 day incubation should be comparable.

The mycelial phase incubated at 25 C for 10 days showed some protein bands by disc electrophoresis. By increasing the incubation period to 20 and 30 days, there was an increased protein production, probably due to greater permeability of the cell membrane or increased autolysis. Surprisingly, strain 184G type A had fewer protein fractions than type B of the same strain at 25 C even after 30 day incubation. The results showed that a strain of H. duboisii and H. capsulatum strain 184G type A and B were all different in electrophoretic patterns after 30 day incubation at 25 C. For 10 day incubation of cultures of H. capsulatum strain G-184 type A and type B at 25 C, the filtrate yielded only 1 homologous band, an indication that type A and type B of the same strain produced the same proteins in the younger cultures. Protein patterns changed after longer

incubation periods. These results agree with those reported by Shechter et al. (1967), who discovered that proteins in the filtrates of 15 and 30 day cultures of C. immitis in asparagine medium were quantitatively and qualitatively different. The difference in electrophoretic patterns of type A and B of the same strain of H. capsulatum was clearly demonstrated after 20 and 30 days of incubation at 25 C and 37 C, while at 10 days incubation, they were the same. Histoplasma duboisii showed common fractions with both H. capsulatum type A and B indicating the close relationship of these two species. When the results of additional strains of both species at 30 day incubation at 25 C are compared, strains of H. capsulatum yielded variable protein patterns. Some were similar, different, or clearly related, indicating the degree of strain differences. In addition, H. duboisii strains B 937, B 939 did not yield identical patterns. When a number of isolates of both species were studied by this method, specific characteristics of electrophoretic mobility of soluble protein in the species could not be determined. Strains of H. capsulatum showed different characteristic protein patterns when compared with each other and with strains of H. duboisii. The interpretation of the difference or similarity of these two types of Histoplasma based on disc electrophoresis is rather difficult.

Shechter et al. (1966, 1968) found that different species of dermatophytes yielded different characteristic protein bands. The number of electrophoretic mobilities of the protein fractions of the 4 isolates of each species were very similar. Their data indicated that the qualitative electrophoretic patterns of these species are relatively stable taxonomic characteristics.

Stipes (1970) also found variation in electromobility of protein extracts from the mycelium of 4 strains in each of three species, Ceratocystis ulmi and C. fagacearum, and C. fimbriata but observed greater similarity in mycelial protein within the isolates of the species. Logically the electrophoretic characteristics of 13 strains of H. capsulatum should be more similar than those of 3 strains H. duboisii if these two species are really different. Stipes has already shown that two species are morphologically different if protein extracts from the two species give different characteristic patterns in disc electrophoresis, on the other hand the patterns of protein extracts should not be much different if the two species are similar morphologically. These references along with our results support the work of Drouhet (1967) and Andrieu et al.(1969) that H. duboisii is very closely related to H. capsulatum, due to the similarity in fractions.

Membrane protein in the yeast-like phase of both species of Histoplasma was applied according to Takayama (1966), Razin and Rottem (1967) using phenol acetic acid water reagent to dissolve the membrane protein in the cells. This was followed by disc electrophoresis with 5M urea in the polyacrylamide gels. The results showed little variation in electrophoretic patterns among strains of both species of Histoplasma and in the same species. Lipman (1970) pointed out that disc electrophoresis of membrane protein used by Razin and Rottem would not differentiate strains of Mycoplasma pneumoniae. This method therefore is useful to differentiate between species rather than between strains in the same species. This was a good tool to study the difference in the electrophoretic mobility between H. capsulatum and H. duboisii since the results of this research showed essentially the same electrophoretic patterns between strains of H. capsulatum and H. duboisii in the yeast phase. This indicates these two are very closely related.

The culture filtrate of the yeast-like phase of the organism from CPYG agar medium incubated at 37 C for 30 days produced electrophoretic patterns that also gave encouraging results. Although strains of H. capsulatum yielded different protein patterns in the yeast phase, common fractions among strains were clearly evident. A strain of H. duboisii gave a protein band

which was not found in strains of H. capsulatum. This protein band was later shown to be a possible PNPGase. This marked difference in the yeast phases in comparison to the mycelial phases of these organisms is significant for the preliminary screening to find the difference in H. capsulatum and H. duboisii that lead to extensive investigation with a number of isolates of both species. The protein patterns from the yeast and mycelial phases of the same strain in the same medium were much different indicating the unique metabolic patterns of the two phases of the organism. This result supports Pine (1957) who hypothesized that the different groups of metabolic reactions occur for each form.

Survey of Enzyme Activities in Liquid and Solid Culture Media of H. Capsulatum and H. Duboisii

The survey of enzyme activities in H. capsulatum and H. duboisii followed the method described by Beneke, et al. (1969) for detecting the diffusible enzymes in different media, both liquid and solid by use of paranitrophenyl substrates.

In the mycelial phase H. capsulatum and H. duboisii when grown in CPYG and BHI with 0.1% cysteine, were not significantly different in enzyme activities with the substrates used. However, in Sabouraud dextrose broth

or agar, the mycelial phase of one strain (MSU) of H. duboisii produced high activity of alpha and beta-PNPGase when the incubation period was increased. In the yeast phase at 37 C again only one strain of H. duboisii produced high activities of alpha and beta-PNPGase in CPYG and PHI with 0.1% cysteine agar media. All but one strain of H. duboisii produced PNP alkaline phosphatase while none was produced by any of the H. capsulatum strains. The strain of H. duboisii at 37 C showed a marked difference in these physiological activities from strains of H. capsulatum. It is possible that H. capsulatum strains in the yeast phase have different metabolic requirements for synthesizing these enzymes, since in the mycelial phase both species produced high activities of alpha and beta-PNPGase in BHI with cysteine and CPYG media. On the other hand the degree of permeability of H. capsulatum specific for alpha and beta-PNPGase in Sabouraud dextrose broth at 25 C and in CPYG and BHI broth at 37 C might be lower than that of the H. duboisii strain.

The organisms were grown on an enrichment medium well supplied with vitamins and amino acids necessary for growth and synthesis of proteins. On this basis the problem of variation in enzyme production due to different nutritional requirements of the organisms should be

minimized. By comparison of different enzymatic activities in the same medium under the same environmental conditions, the enzymatic activities of one organism can be identified as differentiated from the others. The strains of both species produced similar enzymatic patterns. In the yeast phase of all but one strain of H. duboisii produced much higher alpha and beta PNPGase and alkaline PNP-phosphatase than the strains of H. capsulatum. Later, it was found that 3 strains of Histoplasma duboisii produced much higher activities of alpha and beta-PNP-glucosidases in Sabouraud dextrose agar in 30 days at 25 C in the mycelial phase and higher activities of alkaline PNP-phosphatase in CPYG and BHI agar media in 20 days at 37 C in the yeast phase. Although the B-939 strain of H. duboisii did not produce high activities of alpha and beta PNP-glucosidase in the yeast phase, alkaline PNP-phosphatase was high. These differences in enzyme activities of the two species in the yeast and mycelial phase are indications of physiological difference in H. capsulatum and H. duboisii. Previous studies have shown differences in urease production (Coremans, 1963), hydrolysis of amino acid (Rosenthal and Sokolsky, 1965) and hydrolysis of gelatin (Berliner, 1967) when certain strains were used, however recent studies with additional strains have shown variable results (Blumer and Kaufman, 1968).

In regard to physiological difference between Type A and B of the 4 strains of H. capsulatum, Type A of each strain was 2-3 times higher in activities for alpha and beta PNPGase and alkaline PNP-phosphatase than those in type B in BHI medium plus 0.1% cysteine after 20 days at 25 C. These differences supplement the others summarized by Berliner (1967) who was the first to report type A and B occurred in the same strain of H. capsulatum. White, Garrison and John (1970) found chromatographic differences in histoplasmins derived from type A and B of the same strain.

Alpha and beta-PNPGases produced by the MSU strain of H. duboisii in the CPYG medium at 37 C are not produced by H. capsulatum. A further study of the nature of these enzymes was made to obtain a better understanding of the physiology of this organism.

The enzymes alpha and beta-PNPGase were found to be located in the same position in the polyacrylamide gels so they might be similar proteins which are specific for either alpha or beta PNP-glucosidase linkage. Other evidences that supported this assumption were the optimal pH and the activities at high temperature of both enzymes were nearly identical. The optimal pHs for the enzymes were 4.6 and 4.8. The optimal pH of apparently these same enzymes produced by Polyporus annosus was

3.5 - 3.9 (Norkraus, 1957), by Schizophyllum commune was 5.4 (Wilson, 1967), and by Collybia velutipes was 4.6 - 5.6 (Nonknans, 1957). However, in this investigation the enzyme preparation was the culture filtrate instead of using acetone extracted enzyme. Temperature maximum for these enzymatic reactions was 69 C which was relatively high when compared with 52 C for the same enzyme produced extracellularly by Schizonphyllum commune (Wilson and Niederpruem, 1967). Surprisingly, activity of alpha and beta PNPGases from H. duboisii in CPYG medium at 37 C was destroyed completely when held at 60 C for 20 minutes, which is almost similar to PNPGase produced by Collybia velutipes (Norkraus, 1957). This enzyme, produced by Polyporus annosus, was destroyed completely by heat at 80 C for 20 minutes (Norkraus, 1957). An alkaline pH in the culture filtrate of H. duboisii might cause a synergistic effect with the heat in deteriorating enzyme activity, but in the frozen state (-20 C), the alkaline pH showed no effect on enzyme activity after storage of the culture filtrate in the freezer for 90 days, the enzyme activities were still the same as before storage. In an attempt to determine the essential factors for PNPGase production in CPYG medium at 37 C, casamino acids (acid hydrolysate of casein) were substituted for the essential casein in the CPYG liquid medium. None or much lower alpha and beta PNPGase

activity occurred. In the solid medium in which 1.5% agar was added to CPYG medium and when casamino acids were substituted for casein, the activity of alpha and beta PNPGases were detected but lower than the same medium with casein. This evidence indicated that the important factors that induced alpha and beta PNPGase were in the casein. When casein was acid hydrolysed to casamino acids these factors were apparently eliminated by acid hydrolysis. When 1.5% agar (Difco) was added to this medium, some impurities in the agar might function as co-factors for enzyme production as in casein. Such factors could be mineral salts, enzyme substrates, or even amino acids necessary for enzyme synthesis, enzyme activator or a certain kind of vitamin. Generally during acid hydrolysis of protein, tryptophane is destroyed (Lehninger, 1970). Also, after acid hydrolysis of casein, the amount of iron was decreased to 5 micrograms per one gram of casamino acids (Difco Manual 9th Ed., 1953). These substances were thought to be essential for PNPGase production: iron salts such as ferric phosphate, ferric citrate, and ferrous sulphate; amino acids as tryptophan and serine; the enzyme substrate, dextrine, and the salt sodium phosphate. Each was added separately in 1 mg/ml amounts to CPYG liquid medium in which casamino acids were substituted for casein. Standardized inocula were used

in each culture to produce the same amount of the organism. No differences in enzyme activities were detected in the filtrate of the media for PNPGases after 15 days incubation at 37 C. No further attempts were made to determine the essential factors in the medium for enzyme production.

D-cellobiose was found to be the carbohydrate next to glucose that promoted good growth of H. duboisii and was among the best for production of PNPGases in culture. It is a beta glucosidic linkage compound which is possibly a natural substate of PNPGase that is produced by H. duboisii at 37 C. Cellobiose is known to enhance alpha and beta PNPGase of several fungi in the Ascomycetes and the Basidiomycetes (Wilson, 1970).

Glucose is an inhibitor for this enzyme production as the concentration of glucose affected the amount of inhibition when glucose was mixed with PNPGase. This agreed with Wilson and Niederpruem (1967) who found that glucose was the competitive inhibitor of beta-PNPGase produced by Schizophyllum commune. When varying amounts of glucose were added in CPYG medium and the activities of alpha and beta PNPGase were assayed, the highest activities were found when 1.5% glucose was incorporated in the medium. With either increasing or decreasing concentration of glucose, the activities of alpha and beta PNPGases were lower. The concentration of glucose

in the medium controls or regulates the production of these two enzymes. Hauge, MacQuillan and Halvorson (19) also found that when yeasts (Saccharomyces fragilis X S. dobzamskii) were grown in a synthetic medium with 2% glucose, the production of soluble beta glucosidase was repressed over 90% when compared with that in synthetic medium with succinate.

From these results it appears that 1.5% glucose in the medium provided a suitable energy source for the H. duboisii to grow more luxurantly than in the medium containing no glucose or with glucose below 1.5%. If the medium had above 1.5% glucose, the organisms grew abundantly but the excess amount of glucose appeared to repress PNPGase activity and later inhibit PNPGase activity.

In the limited number of ultra thin sections made of the mycelial phase of Histoplasma capsulatum and H. duboisii, the organelles appeared similar except for the Woronin bodies in the former. With a more intensive electron microscopic study of H. duboisii these bodies may be found.

A Possible Differential Medium for Histoplasma capsulatum and H. duboisii

Comparative study of these two organisms led to an attempt to develop a medium in which an observable

change, resulting from a biochemical reaction in the cultures could be used to differentiate H. duboisii from H. capsulatum. Such a medium would be very useful since there is no way to differentiate the two organisms in vitro. This type of medium is relatively difficult as considerable variation occurs among strains of both species.

In the enzyme experiments an unusual reaction occurred in the MSU strain of H. duboisii. Every time the iron salts were added to CPYG agar, the culture of this H. duboisii strain turned a dark purple color after 10 days incubation at 37 C. An assumption then was made that the iron salt in the medium was reduced to form a compound of iron and sulphur, such as ferrous sulfide which was dark purple in color. A reducing agent such as hydrogen sulfide could be produced by the organism in CPYG agar. The combination of ferrous sulphate and sodium thiosulphate were found to be the best hydrogen sulfide detecting agent in bacterial cultures according to Sulkin and Willet (1940). They found incorporation of these agents into CPYG medium which was very good for supporting the growth of H. capsulatum and Blastomyces dermatitidis, should be a useful medium for detecting hydrogen sulfide produced by both organisms. Hydrogen sulfide blackens the agar medium around or under the colonies, since hydrogen sulfide reduces ferrous sulfate producing

a dark color from the sulfur-containing compound. Sodium thiosulphate is the available sulfur source for hydrogen sulfide production (Sulkim and Willet, 1940). The results proved that thio medium was usable. Only the colonies of one strains of H. duboisii (MSU strain) turned purple color while 5 strains of H. capsulatum did not change in color after 14 days incubation at 37 C. The color of colonies of H. capsulatum remained white until the end of the experiment (30 days). It was found later that only ferrous sulfate, added to CPYG agar, was sufficient to develop a purple color in the yeast phase of H. duboisii. On the other hand ferrous sulfate alone, or a combination of ferrous sulfate and sodium thiosulfate, added to BHI agar produced no color change after 30 days incubation at 37 C as occurred in CPYG medium after 10 day incubation. This results indicated that sodium thiosulfate was not the source of sulfur that this fungus used to produce hydrogen sulfide as is the case in some bacterial cultures. The reducing agent such as hydrogen sulfide, if it really existed, probably came from catabolism of organic sulfur in the CPYG medium. Sulfur amino acids as cystine or cysteine when oxidized to form pyruvic acid, one of the pathways is catalized by the enzyme desulfhydrase (Lehninger, 1970). Thus the free SH groups might form hydrogen sulfide in the medium. The growth of H. duboisii at 37 C in BHI agar with either ferrous

sulfate alone or a combination of ferrous sulfate and sodium thiosulfate could not reduce ferrous sulfate. This might be due to a deficiency in the sulfur source of this medium. If this assumption was right the addition of cysteine, one of the essential sources of nitrogen for H. capsulatum (Salvin, 19) and ferrous sulfate in BHI or CPYG agar should induce the organism to produce hydrogen sulfide. Escherichia coli was able to produce hydrogen sulfide from cysteine by means of cysteine sulfhydrase from washed cells and glucose as a stimulating agent (Anderson et al. 1963). For this reason, colonies of Histoplasma strains that produced hydrogen sulfide on both media should turn a purple color after a suitable period of incubation. This results did not occur. All three strains of H. duboisii did not become purple in color in both media after 10 days incubation while all 5 strains of H. capsulatum likewise had no color development at the end of 30 days incubation at 24 and 37 C. This indicated that with excess cysteine in the medium, production of hydrogen sulfide is inhibited. The reason H. capsulatum cannot reduce ferrous sulfate has not been determined, however, there are three pathways in which the process of oxidation of cystine or cysteine to pyruvic acid occurs. One involves

the transfer of SH groups to some other compound by the enzyme transulfhydrase (Lehninger, 1970) so there is never a chance to form hydrogen sulfide. The oxidation of these amino acids by H. capsulatum and some strains of H. duboisii might be similar to the thio pathway.

Most strains of H. capsulatum changed the color of the casamino acid-yeast extract-cellobiose color indicator medium to red more rapidly than strains of H. duboisii in the mycelial phase. It appears that H. capsulatum is able to break down the nitrogenous compounds more readily, releasing ammonia, giving the alkaline reaction in the medium. This reaction is similar to the reactions reported by Taplan et al. (1969) on dermatophytes which produce an alkaline pH in the medium containing soytone while most contaminant fungi do not. When cellobiose was replaced with glucose in the medium, the alkaline pH change occurred more slowly in H. capsulatum for an undertermined reason.

When this medium was used for the yeast phase of both species, alkaline pH changes in the medium was evident for strains of H. capsulatum after 24 hours while most strains of H. duboisii required 48 to 72 hours.

On the basis of the electrophoretic protein patterns between strains of H. capsulatum and H. duboisii, the two species appear very closely related. However, alkaline PNP-phosphatase was consistantly higher in the yeast phase

of H. duboisii with one exception. Even in this exception, the strain produced the typical large sized yeast cells of H. duboisii on CPYG medium at 37 C. Wang et al. (1958) and Pine et al. (1964) indicated presence of larger yeast cells in culture or tissue was significant in H. duboisii. The enzyme studies and electrophoretic protein patterns of a number of strains of H. capsulatum and H. duboisii support the suggestion made by Ajello (1968) that the large yeast cell form of histoplasmosis should be considered H. capsulatum Darling, 1906 var duboisii (Vanbreuseghem, 1952) Ciferri 1960. Additional studies on the enzyme systems, other biochemical activities, and the discovery of the sexual reproduction of H. capsulatum and H. capsulatum var duboisii may lead to a better means of in vitro differentiation.

SUMMARY

1. A comparison was made between the soluble proteins in the culture filtrates of strains of Histoplasma capsulatum and H. duboisii in CPYG, BHI and Sabouraud dextrose liquid and solid media by the disc electrophoresis method. The protein bands showed close relationships between H. capsulatum and H. duboisii, with some variation in patterns among strains of both species.

2. Disc electrophoresis of soluble proteins extracted from culture filtrates after 20 days at 25 C from H. capsulatum strain G 184 type "A" and "B", and H. duboisii MSU strain, produced 1, 8, and 4 fractions respectively. After 30 days, type "A" had 3, type "B", 7, and H. duboisii had 5 fractions. Three fractions of A were common with B, while 3 of the fractions of H. duboisii were common with B, but not homologous to A.

3. Disc electrophoresis of culture filtrates at 37 C of H. capsulatum type A produced only 5 fractions, type B, 2, both of which were homologous with those in type A, while H. duboisii produced 3 fractions, 1 of which was common for type A and B.

4. Disc electrophoresis of phenol acetic acid water cell extract of 2 species grown on CPYG medium at 37 C showed the band patterns were very similar for 9 strains of H. capsulatum and 5 strains of H. duboisii. Histoplasma capsulatum type A and type B had almost identical patterns with a range of 8 to 10 bands.

5. Paranitrophenol substrates were used to assay the culture filtrates and agar media for enzyme activities form H. capsulatum and H. duboisii. In the mycelial phase both species produced high enzyme activities in PNP-alpha- and PNP-beta-D-glucoside substrates. Significantly, H. capsulatum type A had 2 - 8 times higher activity in BHI agar than type B for alpha-and beta-PNPGases. In the yeast phase at 37 C only H. duboisii had high enzyme activities for PNP-alpha and PNP-beta-D-glucoside.

6. The kinetics, inhibitors, optimal temperature, and pH were studied for alpha- and beta-PNP-glucosidases. These two enzymes were found to be located in the same fraction in acrylamide gels.

7. High PNP-alkaline phosphatase was produced by H. duboisii strains in CPYG agar at 37 C while it was low in H. capsulatum strains.

8. The study of the fine structure of the young hyphae of H. duboisii showed similar organelles to those in H. capsulatum as reported in the literature. Woronin

bodies were found near the septa in the hyphae of H. capsulatum, but none in the limited number of sections made for H. duboisii.

9. Many strains fo H. capsulatum changed the pH in the medium more rapidly than most strains of H. duboisii. With a phenol red indicator in the medium, H. capsulatum changed the color of the modified CPYG medium to red while most strains of H. duboisii did not change the yellow color in the medium.

10. The similarities in enzyme studies and electrophoretic protein patterns in the two species appears to support the proposal that the large yeast cell of histoplasmosis should be considered H. capsulatum Darling, 1906 var. duboisii (Vanbreuseghem, 1950) Ciferri 1960.

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