71-12,549

BANNISTER, Edward Ross, 1938-
ISOLATION AND CHEMICAL STUDIES OF SKIN TEST ACTIVE COMPONENTS FROM BLASTOMYCES DERMATITIDIS.

The University of Oklahoma, Ph.D., 1971
Microbiology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ISOLATION AND CHEMICAL STUDIES OF SKIN TEST ACTIVE COMPONENTS FROM BLASTOMYCES DERMATITIDIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

BY

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Norman, Oklahoma

1971

ISOLATION AND CHEMICAL STUDIES OF SKIN TEST ACTIVE COMPONENTS FROM BLASTOMYCES DERMATITIDIS

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGEMENT

The author wishes to express sincere appreciation to Dr. Howard W. Larsh for his suggestions and many hours of discussion concerning this research.

Sincere gratitude is extended to Drs. J. B. Clark, George Cozad, John Lancaster and Eddie Smith for their assistance in preparing the final copies of this manuscript.

A special thanks is extended to Dr. Juneann Murphy for her invaluable aid in the planning and writing of this research.

Special acknowledgement is given to my wife, Margie, for her encouragement throughout this research.

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ISOLATION AND CHEMICAL STUDIES OF SKIN TEST ACTIVE COMPONENTS FROM BLASTOMYCES DERMATITIDIS

CHAPTER I

INTRODUCTION

The disease blastomycosis caused by Blastomyces dermatitidis appears to be similar to coccidioidomycosis and histoplasmosis in that the lungs are the primary locus of infection (2). The other forms of the disease, cutaneous and chronic, result from the dissemination of the fungus from the lungs (45). The geographic distribution of this fungus is not limited to the North American continent as now infections have been found in Africa (13).

Infections of lower animals with this fungus are quite common, with the dog the most susceptible of these animals. Menges (29) reported that there have been 113 canine cases in the United States and three in Canada. Later in a more intensive study 79 canine cases were reported for the state of Arkansas (30). As far as the human population is concerned, it is known that B. dermatitidis infections are prevalent in the United States. A review by Busey (5) showed that in 170 United States Veterans Administration Hospitals,

during the period 1946 through 1957, 198 proved cases of blastomycosis had been diagnosed, an average of 18 cases per year. The disease in North America is not limited to the United States, as cases have been reported in Canada (15).

Despite all the evidence for the prevalence of the disease, the natural habitat of this fungus is not known. There is evidence, however, of isolation of B. dermatitidis from soils in Kentucky and Georgia. The problem associated with these isolations was the fact that after the first isolation the organism could not be isolated a second time (8, 9, 38).

The definite diagnosis of infection with a pathogenic fungus at this time requires either the isolation of the agent or the demonstration of its presence in infected tissue. When fungi invade the human or lower animal body, antibodies of various types may be formed. These antibodies can serve as an indicator of the existence of infection or previous contact with the fungus, but their exact function in the immune process is little understood. Because of these phenomena considerable effort has been directed in the development of serologic techniques and skin testing programs to aid in the diagnosis and prognosis of fungus diseases.

The present knowledge concerning the immune response in humans with blastomycosis is small. The currently available methods of detecting and measuring antibody in blastomycosis are far from perfect, and only rarely is useful

information obtained from either skin testing or serological testing.

A characteristic feature of infection by B. dermatitidis is the development of a hypersensitive state. The reaction is demonstrated by a localized edematous response to the intradermal injection of many different preparations from mycelial and yeast phases. The response reaches its peak in 24-48 hours and then rapidly fades. The broth filtrate of the mycelium (blastomycin) is used almost exclusively for skin testing.

Emmons, Olson and Eldridge (12) have reported nearly complete cross reactions between histoplasmin and blastomycin. Howell (19) has found that the reactions of the guinea pig were dependent upon the particular lot of antigen employed, the critical titer of this antigen and the degree of sensitivity of the animals employed to determine the titer.

Bunnell and Furcolow (4) found that one of ten proved cases of systemic histoplasmosis reacted to blastomycin. However, if the histoplasmin and blastomycin were diluted no cross reaction occurred with blastomycin but histoplasmin was still positive. The homologous antigen still reacted at higher dilutions whereas the heterologous antigen did not react.

Martin's work (28) suggests that H. capsulatum and B. dermatitidis possess common antigens contained within the

yeast cells which are liberated by sonic treatment. However the carbohydrate materials washed off the surfaces of the untreated cells are much more specific, in that only one of the 12 guinea pigs infected with Histoplasma gave a strong reaction to the extract of untreated Blastomyces cells. It was also shown that none of the Blastomyces infected pigs reacted strongly to the extract of untreated Histoplasma cells.

Dyson and Evans (10) isolated fractions from the yeast phase cultures of B. dermatitidis and assayed them for skin test reactivity in infected rabbits and guinea pigs. Somatic antigens extracted from yeast cells did not possess the desired criteria of high sensitivity and specificity; however, a fraction precipitated with 67% ethanol from yeast phase cultural supernate was superior to blastomycin. This fraction has produced a delayed skin reaction in B. dermatitidis infected rabbits in amounts as low as 0.01 ug and failed to react in rabbits infected with H. capsulatum, C. immitis, C. albicans, S. schenckii, and Cryptococcus neoformans. A procedure to remove protein, however, destroyed the skin test reactivity of this antigen.

In a study of skin tests on 134 humans Knight, Coray and Marcus (26) injected histoplasmin, blastomycin and polysaccharides which were derived from broth in which the yeast phase of H. capsulatum and B. dermatitidis had grown respectively. Histoplasmin and polysaccharides from H. capsulatum

were equally reactive. The B. dermatitidis polysaccharide yielded five positive reactions among persons exhibiting concomitant histoplasmin sensitivity.

Knight and Marcus (25) were able to extract specific polysaccharides from yeast phase broth cultures of H. capsulatum and B. dermatitidis. Ten ug of these polysaccharides produced skin test reactions in guinea pigs infected with either H. capsulatum or B. dermatitidis.

Friedman and Conant (15) demonstrated cross reactions between guinea pigs infected with B. dermatitidis and Paracoccidioides brasiliensis.

Peck et al. (36) indicated that on chemical analysis of B. dermatitidis they found at least two distinct polysaccharides. Minute quantities of these polysaccharides fix complement with sera prepared by immunizing rabbits with whole B. dermatitidis. Patients with North American blastomycosis who are in the allergic state of the disease respond to intracutaneous injection of the polysaccharide with an induration which reaches its maximum within 24 hours.

Balows et al. (1) in their study found that dermal sensitivity as an indication of past or present infection with B. dermatitidis is best shown by a heat-killed yeast phase suspension of the organism as the testing antigen. Also the Blastomyces vaccine gave consistently better results than either of two different blastomycin antigens. Skin test reactivity and circulating antibodies to B. dermatitidis were

not regularly occurring features of active blastomycosis. They conclude that so far no valid data can be obtained from skin test surveys employing blastomycin.

It is now generally agreed that the dimorphic fungi comprise a multiplicity of components in both growth phases. Which growth phase contains the broader or more suitable antigen for use in skin testing is still far from clear. According to Kaufman (23), if a reliable skin test antigen for North American blastomycosis is to be produced, it will probably be derived from the yeast form rather than from the mycelial state.

The inability to isolate a reliable serologic or skin test antigen could arise from the problem of reproducing crude preparations of the same uniform and broad antigenic spectrum. Until this can be solved the superior phase of growth antigenically will be difficult to determine. All factors involved such as pH, temperature, duration of incubation can to a great extent be rigidly standardized. The variation in initiating inoculum appears to be one of the most likely causes of antigenic variation in the same and successive crude preparations according to Campbell (6).

Attempts at isolating skin test reactive components of histoplasmin have been done using ion-exchange chromatography (18). Greene's work (18) was corroborated by the findings of Markowitz (27) and Fadula and Larsh (14) using DEAE cellulose. The development of cross-linked dextran

polymers has allowed a new approach to fractionation procedures based on molecular size. Sprouse et al., (42) using using one of the cross-linked dextran polymers (Sephadex G-25), demonstrated that this is a rapid and relatively simple technique for fractionation of large samples of crude histoplasmin with the isolation of skin test reactive components.

The comparative disc gel electrophoresis technique for the isolation and characterization of culture filtrates is being used now to a great extent. This technique was first introduced by Ornstein and Davis (33) and had been employed in similar studies in which a high degree of resolution was introduced. Several other workers have found this technique useful (7, 21, 37). Schechter et al. (39) have applied this in the comparative study of dermatophytes, and it has been employed also in separating various antigenic components of H. capsulatum (44).

Electrophoretic separations of B. dermatitidis have not been reported. There is no reason to assume that crude antigenic preparations of the same strains will not vary quantitatively and possibly qualitatively in antigenic content, regardless of the phase in which they are cultured.

This study is designed to obtain information concerning the production of a skin test active component for B. dermatitidis. Alcoholic precipitation and an ion-exchange resin were used in attempts to isolate skin test active

components. Whether yeast or mycelial phase contains the broader or more suitable antigen for use in skin testing, the chemical nature of this active component and the specificity and sensitivity of this component were investigated.

CHAPTER II

MATERIALS AND METHODS

Skin Testing

1. Animals. Female Hartley strain guinea pigs and white New Zealand rabbits were used for all skin testing. Seventeen guinea pigs were used; 11 were sensitized with Blastomyces dermatitidis (Davis) and 6 were used as controls. Twenty rabbits were used; 11 were sensitized with B. dermatitidis (Davis) and 9 were used as controls. All animals were initially tested with histoplasmin (1:25) and blastomycin (1:10) received from the Center for Disease Control, Atlanta, Georgia and those exhibiting negative reactions (less than 5 mm induration) were used.

2. Preparation of sensitizing dose. The mycelial phase culture for sensitizing the guinea pigs and rabbits was grown for 14 days on Sabouraud's dextrose agar at 27° C. A slant was scraped by adding 10 ml physiological saline to it and the suspension was ground with a teflon grinder. A total count using a haemocytometer and a viability count using standard plate counting techniques of this suspension were used to standardize the infecting dose. The yeast phase

culture for sensitization of the rabbits was grown on blood agar for 4 days at 37° C. The slants were washed with physiological saline and the suspension was centrifuged and washed one time in saline. Total counts using the haemocytometer were made and a viability count using standard plate counting techniques was used to determine the number of viable cells present.

3. Sensitization of animals. An adjuvant for sensitizing the guinea pig consisting of Arlacel A (Hilltop Lab. Inc., Cinn., Ohio) and Marcol 52 (Humble Oil and Refining Co.) were mixed in a ratio of 1:1. This mixture was mixed with the inoculum in a ratio of 1:1 and the mixing was continued until a drop put in a beaker of water did not spread out. This was injected into the footpad and neck of the guinea pig so that the animal received 1 x 10^5 viable particles per injection. The rabbits were sensitized by injecting intravenously 5 x 10^6 viable yeast cells (B. dermatitidis -Davis) per kilogram body weight. Sensitization of other rabbits with other fungi is given in Table 1.

4. Skin test procedure. After five weeks and whenever necessary the animals were clipped and shaved for skin testing. Blastomycin (1:10) and Histoplasmin (1:25) received from the Center for Disease Control, Atlanta, Georgia were used as the standards. For testing 0.1 ml (containing the desired concentration of antigen in Seibert's buffer: sodium phosphate $Na_2HPO_4 \cdot 12\ H_2O$, 4.773 g; potassium phosphate

TABLE 1--Sensitization of rabbits with other fungi

Organism	Inoculation schedule	Route	Dose*	Yeast or mycelium
Blastomyces dermatitidis (242)	Repeated injections over 9 months	IV	1×10^7	Yeast
Blastomyces dermatitidis (Sf.)	Repeated injections over 8 months	IV	1×10^7	Yeast
Aspergillus terreus 1514	Repeated injections over 5 months	IV	5×10^2 to 1×10^4	Mycelium
Aspergillus flavipes 1520	Repeated injections over 5 months	IV	2×10^2 to 4×10^3	Mycelium
Aspergillus carneus 1517	Repeated injections over 5 months	IV	1×10^2 to 2×10^3	Mycelium
Sporothrix schenckii (Parker)	Repeated injections over 5 months	IV	2×10^3 to 6×10^3	Mycelium
Histoplasma capsulatum (Scritchfield)	One injection	IV	2.6×10^8 cells/ kg body weight	Yeast
Histoplasma capsulatum (Scritchfield)	One injection	IB	1×10^6 cells/ animal	Yeast

Sf. = Springfield
IV = intravenously
IB = intrabronchial
* = total number of cells or particles

KH_2PO_4, 0.3627 g; NaCl 8 g and distilled water QS to 1000 ml) was given intradermally on both the rabbit and guinea pig. All antigens tested on all animals were randomized and coded to prevent biased readings. Erythrema (Ery.) and Induration (Ind.) were read after 24 and 48 hours and only the 48 hour readings were used in interpreting the results.

Cultures. The cultures used in this study were obtained from the stock culture collection of the medical mycology division of the Botany and Microbiology Department of the University of Oklahoma. Mycelial cultures were stored at 4° C on Sabouraud's dextrose agar until needed for preparation of inocula. Yeast cells were stored on blood agar at 4° C until needed for preparation of inocula. The following cultures were used in this study: Blastomyces dermatitidis (Davis, 242 and Springfield), Histoplasma capsulatum (Scritchfield), Aspergillus terreus 1514, Aspergillus flavipes 1520, Aspergillus carneus 1517, and Sporothrix schenckii (Parker).

Preparation of antigen

1. Mycelial phase stationary flasks. The inoculum was prepared by growing the fungus (B. dermatitidis -Davis) for 17 days on Sabouraud's dextrose agar at 27° C. The organism was scraped from the slants in 10 ml physiological saline and inoculated, after counting in a haemocytometer chamber, into Smith's asparagine medium (40). Viability studies were carried out on the inoculum using the standard plate count

method. Three 500 ml Erlenmeyer flasks containing 250 ml asparagine medium were inoculated with 1 x 10^5 viable particles/ml and incubated at 27° C at 75% humidity for 17 days. The contents of these three seed flasks were used to inoculate 24 liters of asparagine medium. This inoculated medium was dispensed in liter amounts into two liter flasks and these inoculated flasks were incubated at 27° C with 75% humidity. An uninoculated control flask was included.

At weekly intervals for the first month and monthly intervals thereafter, one flask was removed from the incubator and merthiolate was added to give a final concentration of 1:10,000. After two days storage at 4° C the medium was filtered twice through a Seitz filter. The filtrate which was bottled in sterile antigen bottles in approximately 100 ml amounts and stored at 4° C was the crude antigen used in skin testing. A portion of each antigen was dialyzed at 4° C against distilled water for one week with daily changes of the water. The non-dialyzable material was lyophilized and stored in screw cap tubes at 4° C until used. One bottle of each age also was dialyzed at 4° C against distilled water for one week with daily changes of the water and this material was concentrated by perevaporation at room temperature to 2 ml. It was stored at 4° C until used.

2. Yeast phase. The yeast cells were grown at 37° C on blood agar for 4 days when an inoculum needed to be prepared. The inoculum (B. dermatitidis-Davis) was grown in

neopeptone dialyslate broth (NPD) (10) and tryptose phosphate broth (TP) for three days. The flasks were incubated at 37° C and shaken at 180 rpm on a Controlled Environment Incubator Shaker. Each of 12 two liter Erlenmeyer flasks containing 1 liter of neopeptone dialyslate broth and 12 two liter Erlenmeyer flasks containing 1 liter of tryptose phosphate broth were inoculated so that each flask had a concentration of 3×10^3 viable cells/ml which had been grown in the same medium. The flasks were incubated at 37° C and shaken at 180 rpm on a Controlled Environment Incubator Shaker. After 6 days incubation a final concentration of 1% phenol was added and the flasks were refrigerated for two days. The contents of each flask were centrifuged and the supernatant and the yeast cells were separated and saved for skin testing and chemical studies.

The supernatant of the yeast cells was extracted by the following procedure of Dyson and Evans (10). The supernatant was reduced to approximately 0.5% of original volume by perevaporation at room temperature and acetic acid and sodium acetate were added to give a final concentration of 1% and 10% respectively. The solution was cooled to 5° C and absolute ethanol at 5° C was added and with constant mechanical stirring the precipitate occurring after the addition of 50% ethanol was removed by centrifugation. Additional alcohol at 5° C was added to the remaining supernatant to obtain a final concentration of 67% ethanol. The

precipitate occurring after addition of 67% ethanol was centrifuged and dissolved in water and acetic acid and sodium acetate were again added to final concentrations of 1% and 10% respectively; this was precipitated by the addition of ethanol to give a final concentration of 67%. The precipitate was removed by centrifugation and dissolved in a minimal amount of water and stored at 4° C until used.

The cells saved from the centrifugation were broken and extracted according to the following procedure. The cells were washed three times in physiological saline and a 20% suspension of cells in saline was made. To this suspension glass beads (0.25-.30 mm) were added and this was disrupted in a Bronwill cell disintegrator for 1 minute. The disrupted cells and glass beads were removed and placed in a centrifuge tube and allowed to stand at 4° C for 24 hours. This was centrifuged at 20,000 rpm for 2 hours and the supernatant was saved. The supernatant was dialyzed against distilled water for 2 days at 4° C and extracted by the above procedure of Dyson and Evans (CFE) (10).

Fractionation of antigen

1. Polyacrylamide disc gel electrophoresis. The lyophilized antigen was resuspended in a minimal amount of water (usually 100 lambda). This material and the material concentrated by perevaporation were fractionated by polyacrylamide disc gel electrophoresis using the anionic system according to Buchler instruments manual (3). The gels were

stained with Amido Black for 1 hour followed by partial destaining in 7% acetic acid to yield qualitative protein identification. Duplicate samples were stained with Schiff's reagent for carbohydrate determinations.

2. Dowex 50 (H^+). The precipitate extracted by 67% alcohol from neopeptone dialyslate broth was further fractionated on a 10 cm x 9 mm column of Dowex 50 (H^+), 200-400 mesh, 4% cross linked. The sample was first dialyzed two days against Seibert's buffer at 4° C with changes in the buffer every 8-12 hours. Approximately 130 ug of protein (0.2 ml) were put on the column and eluted with citrate phosphate buffers of pH 3.65 and 6.4 with fractions being collected every minute. The sample as it came off the column was monitored on a Canalco Model DA Widetrack 85 UV Flow Analyzer. The fractions were pooled into three pools and these were dialyzed against physiological saline on a rotary dialyzer at 4° C for two days with changes of saline every 8-12 hours. After the pH was adjusted to 7.3 each pool was used as a skin test antigen and was characterized chemically.

Chemical characterizations

1. Protein determinations. These determinations were made using the modified Folin-Phenol method of Oyama and Eagle (34) and the Micro-Biuret technique (26).

2. Carbohydrate determinations. These determinations were made using anthrone reagent (31), Somagi procedure (41), Bial's reagent (24) and the Lasker and Enkelwitz test (24).

3. Acid hydrolysis for amino acids. (44) Heavy walled pyrex tubes washed several times with acid and stored in an evacuated desiccator were used in the hydrolysis procedure. The precipitate extracted by 67% alcohol from the neopeptone dialyslate broth (625 ug protein) and the skin test active pools (19.5 ug protein) collected from the Dowex 50 column were suspended in tubes in 1 ml of 6 N double glass distilled HCl. The tubes were frozen, evacuated to a pressure of 50 microns and the solutions were thawed until the vacuum was 60 microns. Again the tubes were immersed in the freezing solution which permitted the air bubbles to escape. Gentle shaking of tubes was maintained throughout the freezing and thawing. Finally tubes were sealed at a pressure of 50 microns. The hydrolysis was conducted at 110 ± 1° C for 24 hours. After the tubes had been cooled to 25° C, the HCl was removed in a vacuum desiccator over separate basins of potassium hydroxide and phosphorous pentoxide. The dry residue was redissolved in 0.5 ml water and this was the material employed in amino acid determinations.

4. Acid hydrolysis for carbohydrate. (44) The precipitate extracted by 67% alcohol from neopeptone dialyslate broth (19 ug carbohydrate) and the skin test active pool (6 ug carbohydrate) collected from the Dowex 50 column were resuspended in 2 ml and 1.5 ml respectively of 0.25 N H_2SO_4. They were sealed in tubes by the same process used for sealing in hydrolysis of amino acids. The hydrolysis was conducted at 105° C for 10 hours. After the hydrolysis, the

solutions were neutralized with $BaCO_3$ to pH 7, which was measured by pH paper. The precipitate was removed by centrifugation, the supernatant was evaporated on a rotary evaporator and redissolved in water and this material was used for determination of carbohydrates.

5. Chromatography. Thin layer chromatography using Silica Gel G (1:2, absorbent: water) was used to aid in identification of amino acids and carbohydrates. Two-dimensional and one-dimensional chromatography were used for the amino acids. The solvents used in the two-dimensional were (1) butanol/glacial acetic acid/water (4:1:1) and (2) phenol/water (75:25). For one dimensional the solvent used was methylethylketone/pyridine/water/glacial acetic acid (70:15:15:2). Ninhydrin (0.5 g ninhydrin, 100 ml n butanol and 3 ml glacial acetic acid) was used as the spray for all amino acids. (35) The solvents used for identification of carbohydrates were (1) methylethylketone/glacial acetic acid/methanol (60:20:20) and (2) ethyl acetate/isopropanol (65:35). Aniline phthalate and periodate-benzidine spray were used as sprays for the carbohydrates. (43)

CHAPTER III

RESULTS

Skin testing. Table 2 gives the results of skin testing rabbits with crude mycelial filtrate, CFE precipitate, precipitate from tryptose phosphate broth and precipitate from NPD. Any antigen which gave a 5 mm or more induration was considered positive. In Table 2 it is evident that almost all antigens tested reacted on some of the rabbits. The blastomycin (1:100) did not react and this concentration was assumed to be too dilute to delineate sensitized animals. The crude mycelial filtrate antigen prepared by perevaporation appears to be more sensitive than the one prepared by lypohilization. The antigens prepared from yeast cells and from yeast cell culture filtrate by precipitation with alcohol also give good reactions on sensitized animals.

Cross reaction studies were important in analyzing skin test material. Results using rabbits sensitized with H. capsulatum are given in Table 3. These results indicate that the antigens prepared from yeast cells (NPD precipitate and CFE precipitate) do not react on rabbits sensitized with

TABLE 2--Skin test reactivity in rabbits injected with Blastomyces dermatitidis (Davis)

Antigen		Concentration of carbohydrate (ug)/ test	Skin test reactions in injected rabbits			Skin test reactions in control rabbits		
			Reactors Total	Average of Ery.	Ind.	Reactors Total	Average of Ery.	Ind.
Mycelial								
0 (uninoculated)	L	0.86	0/11	0	0	0/9	0	0
7 months	L	1.12	4/11	10.2	6.5	0/9	0	0
12 months	L	10.0	1/11	12.0	9.0	0/9	0	0
14 months	L	10.4	4/11	10.8	5.5	0/9	0	0
0 (uninoculated)	P	3.4	0/11	0	0	0/9	0	0
7 months	P	1.09	7/11	10.4	7.8	0/9	0	0
12 months	P	2.7	4/11	12.3	9.3	0/9	0	0
14 months	P	9.9	5/11	13.0	9.8	0/9	0	0
Yeast								
CFE precipitate		1.06	4/11	13.0	9.2	0/9	0	0
NPD precipitate		20.4	7/11	15.3	10.3	0/9	0	0
TP precipitate		61.3	10/11	12.3	10.9	0/9	0	0
Blastomycin (1:100)			0/11	0	0			

Ery. = erythrema
Ind. = induration
L = antigen concentrated by lyophilization from crude mycelial culture filtrate
P = antigen concentrated by perevaporation from crude mycelial culture filtrate
NPD = precipitate extracted with 67% alcohol from neopeptone dialyslate broth of yeast
TP = precipitate extracted with 67% alcohol from tryptose phosphate broth of yeast
CFF = precipitate extracted with 67% alcohol from cell-free extract of yeast

TABLE 3--Skin test reactivity of rabbits injected with Histoplasma capsulatum

Antigen	Concentration of carbohydrate (ug)/test	Skin test reactions in rabbits		
		Reactors	Average of	
		Total	Ery.	Ind.
Mycelial				
7 months P	1.09	4/4	10.5	9.7
12 months P	1.02	3/4	11.3	11.6
14 months P	1.6	3/4	11.0	9.7
7 months L	1.12	2/4	11.5	11.0
12 months L	1.36	4/4	12.0	12.5
14 months L	1.96	4/4	12.2	12.2
Yeast				
NPD precipitate	1.06	1/4	5.0	2.0
CFE precipitate	1.06	0/4	0	0
Histoplasmin (1:25)		4/4	19.0	18.6
Blastomycin (1:10)		4/4	14.7	14.0

Ery = erythrema
Ind = induration
L = antigen concentrated by lyophilization from crude mycelial culture filtrate
P = antigen concentrated by perevaporation from crude mycelial culture filtrate
NPD = precipitate extracted with 67% alcohol from neopeptone dialyslate broth of yeast
CFE = precipitate extracted with 67% alcohol from cell-free extract of yeast

H. capsulatum. However, the antigen prepared from the Blastomyces mycelial culture filtrate reacted on these same rabbits. Blastomycin (1:10), one of the standards, also reacted on these rabbits.

In addition to the cross reactions studies in animals sensitized with H. capsulatum, the Blastomyces antigens were also tested on animals sensitized with other fungi. These results are given in Table 4. The antigen prepared from the yeast phase (NPD) only reacted on rabbits sensitized with other strains of B. dermatitidis. The antigen prepared from the mycelial culture filtrate by perevaporation reacted only on B. dermatitidis rabbits, but the lyophilized antigen from mycelial culture filtrate reacted on rabbits with all three fungi.

Comparative titration of the yeast phase antigens is given in Table 5. Both Blastomyces sensitized animals and control animals reacted to the TP antigen. The antigen prepared from NPD was very reactive on sensitized rabbits at a concentration of 1 ug of carbohydrate, but at lower concentration (0.1 ug) the sensitivity was reduced. The antigen prepared from NPD did not react on uninfected control rabbits.

Fractionation of NPD precipitate on Dowex 50 was used to further aid in chemical characterization of the antigen. Table 6 gives the results of skin testing the three pools collected from this column. It appears that only pool 3 is active on Blastomyces sensitized rabbits. The other two pools

TABLE 4--Reactivity of mycelial and yeast phase antigens on injected rabbits

Antigen	Concentration of carbohydrate (ug)/ test	Skin reactions in injected rabbits					
		Aspergillus sp.		*S. schenckii*		*B. dermititidis*	
		R/T	AI	R/T	AI	R/T	AI
Mycelial							
12 months P	1.02	0/3	0	0/2	0	0/3	0
14 months P	1.60	0/3	0	0/2	0	1/3	1
12 months L	1.36	2/3	0	1/2	5	0/3	0
14 months L	1.96	0/3	0	0/2	0	1/3	11
Yeast							
NPD precipitate	1.06	0/3	0	0/2	0	2/3	6
Histoplasmin (1:25)		0/3	0	0/2	0	1/3	2
Blastomycin (1:10)		0/3	0	0/2	0	0/3	0

R/T = reactors / total
AI = average induration
P = antigen concentrated by perevaporation from crude mycelial culture filtrate
L = antigen concentrated by lyophilization from crude mycelial culture filtrate
NPD = precipitate extracted with 67% alcohol from neopeptone dialyslate broth of yeast

TABLE 5--Comparative titrations of yeast phase antigens in injected and control rabbits

Antigen	Concentration of carbohydrate (ug)/ test	Skin test reactions in injected rabbits			Skin test reactions in control rabbits		
		R/T	Average of Ery.	Ind.	R/T	Average of Ery.	Ind.
TP precipitate	61.3	10/11	14.0	11.0	1/9	12.0	2.0
	10.2	5/11	10.2	8.0	2/7	11.0	7.5
	1.02	3/11	11.3	8.0	0/7	0	0
NPD precipitate	10.6	7/11	14.4	12.1			
	1.06	5/11	13.2	10.4			
	0.104	3/11	4.0	2.0			
Blastomycin (1:10)		2/11	8.5	1.5			

R/T = reactors / total
Ery = erythrema
Ind = induration
TP = precipitate extracted with 67% alcohol from tryptose phosphate broth of yeast
NPD = precipitate extracted with 67% alcohol from neopeptone dialyslate broth of yeast

TABLE 6--Reactivity of the NPD precipitate after fractionation on Dowex 50 column

Antigen	Concentration of carbohydrate (ug)/test	Skin test reactions in injected rabbits		
		Reactors	Average of	
		Total	Ery.	Ind.
Pool 1	3.0	0/7	0	0
Pool 2	2.0	0/7	0	0
Pool 3	6.0	3/7	6.6	5.6
Blastomycin (1:10)		6/7	8.5	4.7

Ery. = erythrema
Ind. = induration

did not react on any of the rabbits. Control rabbits were all negative.

Further evaluation of the antigens was carried out using guinea pigs as skin test animals. In Table 7 the results of these tests are recorded. The NPD precipitate (yeast cells) was reactive on sensitized guinea pigs as well as the antigen prepared from the crude mycelium filtrate.

Changes in the growth medium of B. dermatitidis.

Figure 1 shows the variation in concentration of total protein, total carbohydrate and reducing sugars over the 14 month incubation period. A gradual increase in total protein was noted during the first 10 months followed by a greater increase from 10 months to 14 months. The total protein concentration changed from 0.6 mg/ml to 1.5 mg/ml. In contrast, after an initial increase and reduction, total carbohydrate remained fairly stable for 10 months and then decreased rapidly during the remainder of the incubation period. Reducing sugar concentration, after an initial increase and reduction, decreased again within 2 months. These sugars remained fairly stable until the 10th month and then decreased rapidly during the remainder of the incubation period.

Fractionation of antigen

1. Polyacrylamide disc gel electrophoresis. A characteristic electrophoretic pattern for the protein was obtained for the lyophilized and perevaporated samples of the crude mycelial filtrate. Figure 2 shows the differences in

TABLE 7--Skin test on guinea pigs injected with Blastomyces dermatitidis (Davis)

Antigen	Concentration of carbohydrate (ug)/test	Skin test reactions in injected guinea pigs		
		Reactors	Average of	
		Total	Ery.	Ind.
Mycelial				
12 months P	10.2	3/7	9.3	6.6
14 months P	10.6	2/6	8.5	8.0
12 months L	11.3	3/7	7.6	5.6
14 months L	11.4	2/6	11.0	9.0
Yeast				
NPD precipitate	10.8	3/5	8.3	7.6
Blastomycin (1:10)		4/11	8.0	9.0

Ery. = erythrema
Ind. = induration
P = antigen concentrated by perevaporation from crude mycelial culture filtrate
L = antigen concentrated by lyophilization from crude mycelial culture filtrate
NPD = precipitate extracted with 67% alcohol from neopeptone dialysate broth of yeast

Fig. 1.--Changes in total protein, total carbohydrate, and reducing sugars during a 14 month incubation period of B. dermatitidis (Davis).

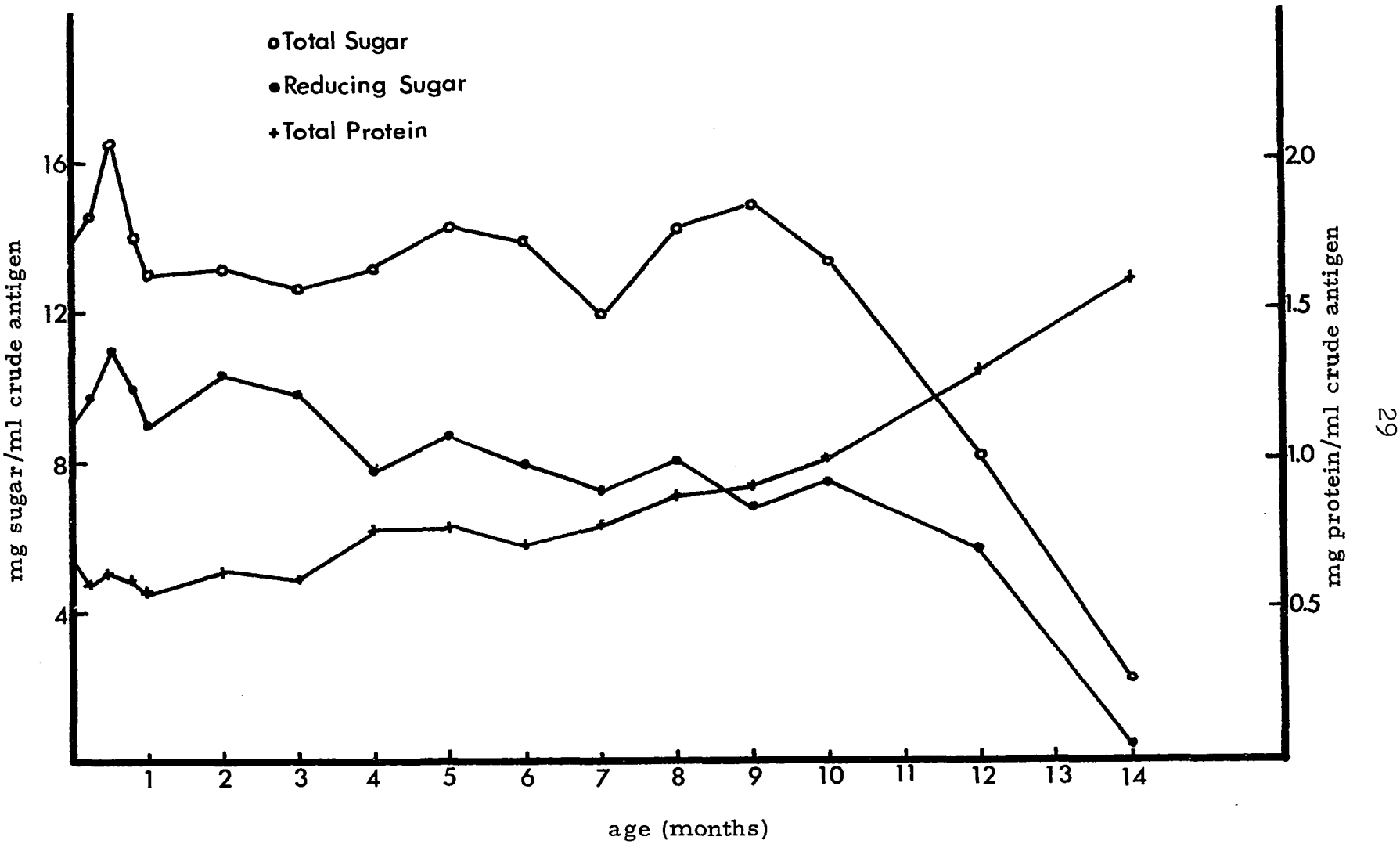
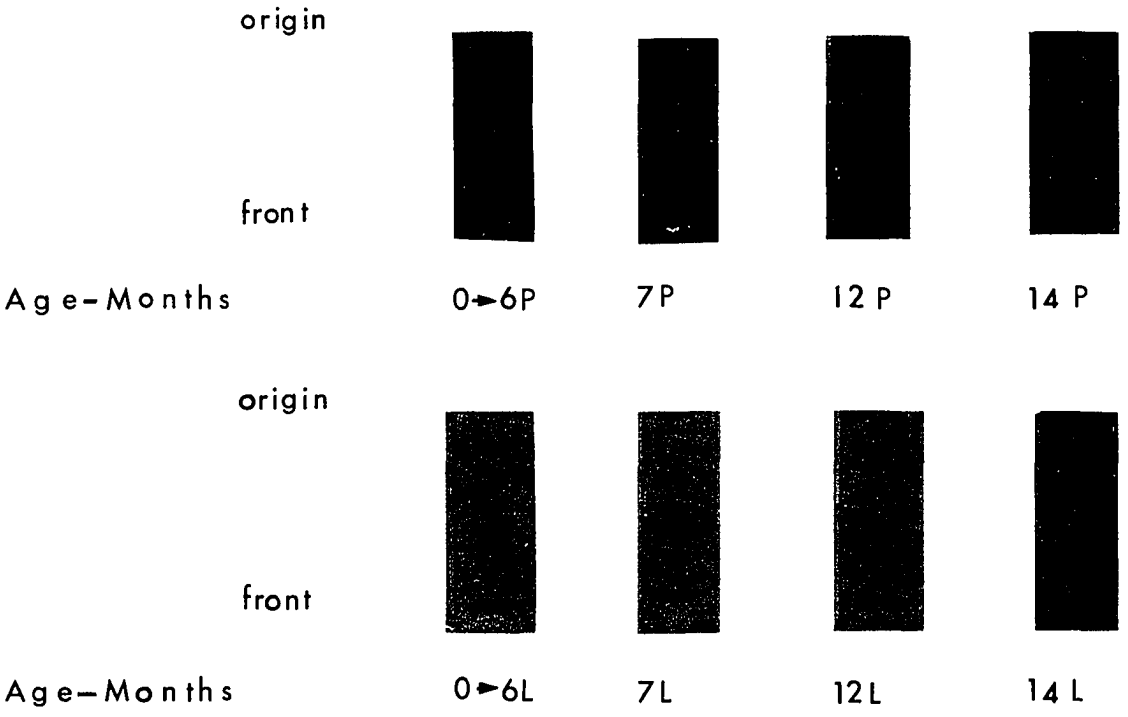

oTotal Sugar
•Reducing Sugar
+Total Protein
mg sugar/ml crude antigen
mg protein/ml crude antigen
16
12
8
4
2.0
1.5
1.0
0.5
1
2
3
4
5
6
7
8
9
10
11
12
13
14
age (months)

Fig. 2.--Electrophoretic pattern in acrylamide gels of B. dermatitidis (Davis) mycelial culture filtrates.

AMIDO BLACK



SCHIFF'S



P = antigen concentrated by perevaporation
L = antigen concentrated by lyophilization

ages between the techniques of preparing the antigen. After 7 months the first bands appeared, and this is when a great difference between the lypohilized and perevaporated samples occurred. In older cultures some of the bands disappeared. The results of the staining with Amido Black and Schiff's reagent indicated some of the bands may be glycoprotein.

2. Dowex 50 (H^+). Figure 3 gives the results of the fractionation of the NPD precipitate on a Dowex 50 column. The fractions were separated into 3 pools from the tubes according to the three large peaks in Figure 3. Pools 1 and 2 were eluted with citrate phosphate buffer of pH 3.65 and pool 3 was eluted with citrate phosphate buffer of pH 6.4.

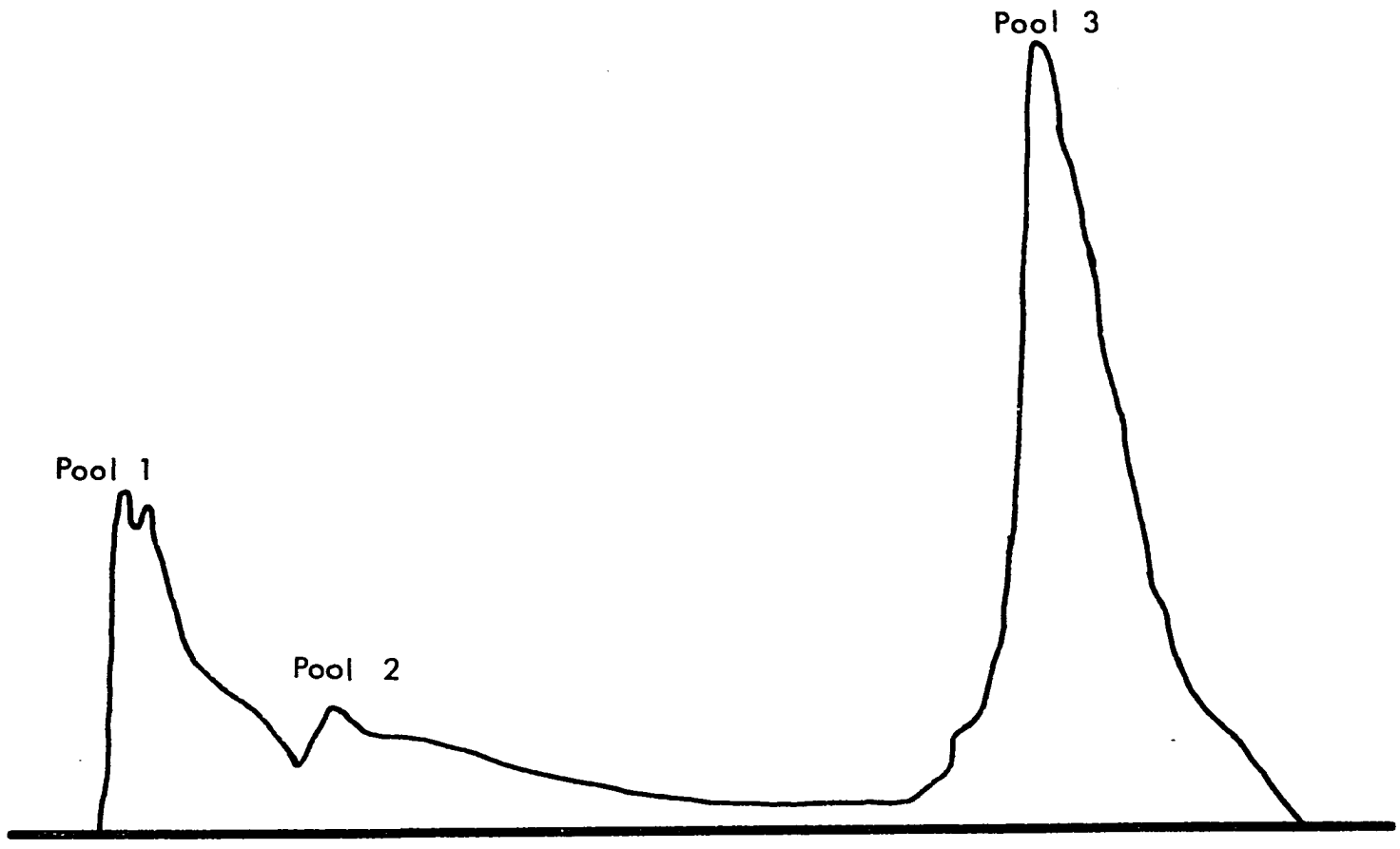
Chemical characterizations

1. Protein. Using the Folin-Phenol reagent the NPD precipitate had 625 ug/ml, but using the micro/biuret test the precipitate had 440 ug/ml. Protein determination on pool 3, using Folin-Phenol reagent, indicated this contained 32.5 ug/ml.

2. Carbohydrate. Total carbohydrate in the NPD precipitate was 190 ug/ml. Bial's test for pentoses was positive and Lasker and Enkelwitz test for ketoses was negative.

3. Chromatography for amino acids. Following hydrolysis of pool 3 and using one-dimensional thin layer chromatography two amino acids or peptides were separated. One was a yellow spot (Rf 0.97) and the other was a violet spot (Rf 0.83). After hydrolysis of the NPD precipitate, six

Fig. 3.--Fractionation of the neopeptone dialyslate precipitate from yeast culture filtrate on a Dowex 50 (H^+) column.


Pool 3
Pool 1
Pool 2

spots were separated using two-dimensional thin layer chromatography.

4. Chromatography for carbohydrates. Hydrolysis of the NPD precipitate and pool 3 were carried out using 0.25 N H_2SO_4. Following hydrolysis of NPD and pool 3 only one spot could be found. This unknown spot had an Rf value close to that of fructose and ribose.

CHAPTER IV

DISCUSSION

Antigens present in crude supernate from mycelial cultures are currently used for demonstrating delayed hypersensitivity to the fungi producing deep-seated infections. The reasons are many as to why it would be desirable to isolate the skin test antigens in pure form. The possession of a pure antigen would permit its chemical structure to be determined, permit the compound to be standardized on a weight basis, and would possibly eliminate some of the undesirable cross reactions occurring among H. capsulatum, B. dermatitidis, C. immitis and other fungi.

The problems that are defined in the isolation of a reliable skin test antigen from B. dermatitidis provided the impetus for this research. Evidence presented in this paper shows that a sensitive and specific skin test antigen can be isolated from the supernate of yeast cultures.

Earlier workers have attempted to isolate a reliable skin test antigen from B. dermatitidis, but whether this antigen should come from the yeast or mycelial phase is still debatable. It seems likely that a more reliable antigen would

be isolated from the yeast since this is the form in which the organism exists in infected tissue. If the mycelium is the infective phase, then during the conversion of mycelium to yeast in tissue a great many different type compounds able to invoke antibody formation would be present and these antibodies would be more likely to react with a mycelial antigen.

The results in this study of testing sensitized rabbits (B. dermatitidis) with homologous antigens indicates that the yeast and mycelial phase can be used to prepare skin test antigen. Each type antigen was reactive and gave good reactions on sensitized rabbits. The antigens prepared from the yeast phase (CFF, TP and NPD) do not apparently give any better reactions than the ones prepared from the mycelial phase by perevaporation. The mycelial antigens prepared by lyophilization gave smaller reactions with some ages and this may be an indication that lyophilization altered the skin test active material in these antigens.

Not only was it considered important in this study that the antigens be sensitive for skin testing but it was just as important that they be specific. Most cross reactions that do occur with B. dermatitidis antigens do so in animals infected with H. capsulatum. Other fungi also are important in cross reaction studies and are necessary for a more complete evaluation. The yeast phase skin test antigen isolated (NPD precipitate) was specific for B. dermatitidis (Davis) sensitized rabbits and the reactions on other rabbits

sensitized with other strains of B. dermatitidis also are considered important. All infections with this fungus are not by the same strain and an antigen such as this would perhaps be of use in epidemiology studies. The mycelial phase antigens were not specific for B. dermatitidis rabbits as reactions on H. capsulatum animals were equal to or greater than the ones received on homologous animals. The problem of cross reactions is present with this type antigen and the presence of similar antigens in these organisms most likely accounts for these cross reactions.

The use of a known amount of skin test material to elicit a desired reaction is as important with the systemic fungi as it is with Mycobacterium tuberculosis. If this could be quantitated further and a specific amount of carbohydrate or protein or combination of these for each skin test used instead of using a known dry weight, the reactions might be more sensitive and specific. This study has shown that as little as 1 ug of carbohydrate from the NPD precipitate can be used to obtain a positive reaction, but using 0.1 ug the reaction is not positive. Since protein was present with both concentrations of carbohydrate, perhaps the right combination of protein and carbohydrate would be ideal for a skin test material.

Even though a positive skin test reaction was obtained with the NPD precipitate, further purification appeared essential since it is known that an alcohol precipitate such

as this might contain many types of protein-carbohydrate complexes. Use of a Dowex 50 column proved efficient in separating and purifying this material. The three pools which were collected were skin tested on B. dermatitidis sensitized animals and only pool 3 proved to be active. Even though the reaction was not as great as had been with the unfractionated sample, undesirable cross reactions may have been eliminated using this method of separation.

In addition to rabbits it was considered desirable to evaluate the antigens in guinea pigs. Both antigens (NPD precipitate and mycelial phase culture filtrate) reacted on sensitized guinea pigs but the reaction with the NPD precipitate was not as striking as it was in the rabbit. It is not possible from these results to evaluate whether the antigen prepared by lyophilization or perevaporation was more sensitive. The difference in these two animals probably reflects a species difference or may be due to the fact that the two species were sensitized by different routes. At any rate, the excellent results obtained with the rabbit may not hold true for other species.

The studying of biochemical changes in the growth medium of a fungus along with skin testing this crude filtrate over a period of months might aid in determining the optimum time to harvest the crude filtrate for use in skin testing. There is no evidence now available as to the biochemical behavior of the mycelial phase of B. dermatitidis in a

synthetic medium. Goodman and Larsh (16) showed that an increase in skin test activity parallels the increase in protein content of crude filtrate in which H. capsulatum had grown. No correlation of this type can be made from this study because not all ages were skin tested. The earliest age tested was 7 months and positive reactions were obtained with this age. Total carbohydrate and reducing sugars show a reduction at the same time that total protein shows an increase, but at this time no significance can be given these results. It does appear that B. dermatitidis in its biochemical behavior in a synthetic medium measuring changes in total protein, total carbohydrate and reducing sugars resembles H. capsulatum (44).

The use of polyacrylamide disc gel electrophoresis in separating the proteins of different organisms has been reported (7, 21, 40, 44), but the separation of the antigens of B. dermatitidis using this technique has not been reported. Using two methods of concentration the crude mycelial filtrate was prepared for electrophoretic separation and skin testing. These methods were found to differ in their action on the crude mycelial filtrate. It appeared that the antigen prepared by perevaporation did not undergo as many changes as the one prepared by lypohilization. It could be that lypohilization of this crude antigen has in some way altered the protein of this filtrate, whereas perevaporation, a less drastic treatment, does not cause this change. It was noted that at

least one of the bands appeared to be glycoprotein from the staining of the gels, as has been found with H. capsulatum (44). Whether the band that appears to be glycoprotein is active as a skin test antigen is not known at this time.

Previous workers in this field have isolated polysaccharide skin test material (28, 13, 25, 23), but few of these attempted to characterize their material as to what carbohydrate is present and if protein is present what amino acids might be there. Chemical studies of the NPD precipitate and pool 3 in this study were enhanced by using thin layer chromatography.

The results from the thin layer chromatography for amino acids indicated that associated with the NPD precipitate were 6 amino acids or peptides, but in pool 3 only 2 amino acids or peptides were separated. The identification of the carbohydrate by chromatography indicated there was only one spot with the NPD precipitate and with pool 3. This unknown spot had an Rf value close to that of fructose and ribose; since chemical tests indicated this carbohydrate was a pentose the sugar present appears to be ribose. Further characterizations and purifications must be done in order to give a final identification of the carbohydrates and amino acids. What type association this is between the carbohydrate and protein is not known, but up to the present two types of linkages have been established for glycoproteins. One involves an N-acylglycosylamine, formed by binding C-1

of N-acetylglucosamine directly to amide nitrogen of asparagine. The second linkage is that in which the beta-carbon atoms of serine and theonine are joined by O-glycosidic linkage to an N-acetylhexosamine residue. There are suggestions for other linkages but these are only suggestions (17).

CHAPTER V

SUMMARY AND CONCLUSIONS

In this study a specific skin test antigen was isolated by precipitation with 67% ethanol from yeast phase culture filtrates. This antigen was reactive in amounts as low as 1 ug carbohydrate/test. Using a Dowex 50 column this material was separated into 3 pools and pool 3 was found to be skin test active. Chemical studies not only of the unfractionated sample but also pool 3 indicates this material is glycoprotein. Carbohydrate results indicate the sugar present appears to be ribose in the unfractionated sample and in the skin test active pool. Protein analysis of amino acids shows 6 amino acids or peptides were separated in the unfractionated sample and 2 amino acids or peptides were separated in pool 3.

Antigens of the mycelial phase culture filtrate were found to be reactive on homologous sensitized animals but cross reactions did occur on rabbits sensitized with H. capsulatum. Results of electrophoretic separation of crude mycelial filtrate and skin test results may be an indication

that lyophilization causes changes in the protein of this filtrate that perevaporation does not.

The growth of B. dermatitidis in a synthetic medium was found to resemble H. capsulatum (44) in its biochemical behavior when measuring changes in total protein, total carbohydrate and reducing sugars.

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