FUNGAL ANTIGENS AND FUNGAL DISEASE: AN ALKALI-SOLUBLE, WATER SOLUBLE ANTIGEN FROM *COCCIDIOIDES IMMITIS* AND COCCIDIOIDOMYCOSIS

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Diagnostic medical mycology has been slow to advance due to a lack of species specific antigens in organisms which cause serious diseases in man. Toward this end, an HPLC analysis was done of the following fungal antigens: histoplasmins HKC-43 and H-42, blastomycin KCB-26, an alkali-soluble, water-soluble antigen from Blastomyces dermatitidis (b-ASWS), a coccidioidin prepared from a toluene lysate of the mycelial-arthroconidia phase of Coccidioides immitis, and an alkali-soluble, water-soluble antigen from Coccidioides immitis (c-ASWS). The HPLC survey included size-exclusion chromatography (SEC), ion exchange chromatography (HPIEC), and reverse-phase chromatography (RP). Resolution was poor with both SEC and HPIEC but was excellent with RP chromatography. The use of RP will allow sufficient separation for further antigenic and structural analysis.

A fungal antigen of importance is the alkali-soluble, water-soluble antigen extracted from the mycelial-arthroconidial phase of C. immitis. A preliminary structural analysis revealed that this antigen contains 55% carbohydrate, 26% protein, and 4.2% lipid on a weight percent basis. It was determined that the carbohydrates present were mannose, galactose, and 3-O-methyl mannose in a 7.6:1.6:1 molar ratio. Chromatography in guanidine-HCl demonstrated four components that contained both carbohydrate and protein. Two of the components had molecular weights > 2×10^5, whereas the other two had molecular weights of 59,485 and 32,000, respectively. It is felt that these components are mannans with side chains ending in galactose, 3-O-methyl mannose, or small peptide groups.

Diagnosis of coccidioidomycosis is difficult. Serum samples from thirty-seven patients with proven coccidioidomycosis were analyzed by GLC for the presence of 3-O-methyl mannose; a sugar unique to the cell wall of C. immitis. This unique methylated sugar was present in all thirty-seven sera tested. Analysis of sera from controls and from patients with aspergillosis, blastomycosis, candidiasis, and histoplasmosis did not reveal the presence of 3-O-methyl mannose. Therefore, 3-O-methyl mannose can serve as a unique marker for the diagnosis of coccidioidomycosis by GLC.

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CHAPTER I

INTRODUCTION

In 1892 Wernicke (134) and Posadas (103) independently described a fatal granulomatous disease in an Argentine soldier. Shortly thereafter, similar cases (104) were recognized in Portuguese farm laborers who had worked in the San Joaquin Valley of California. The causative agent was isolated and identified at this time. Approximately 40 years elapsed between the first description of virulent coccidioidomycosis and the description of a more commonly occurring mild form of the disease.

The salient features of the infection can be briefly summarized. The etiologic agent is Coccidioides immitis, a dimorphic fungus which is restricted to the Western Hemisphere. The organism exists in the soil in its saprophytic form where it develops an extensive mycelial system and produces large numbers of arthroconidia. It is through the inhalation of dust-borne arthroconidia that coccidioidomycosis develops as the primary form of the disease. The spores enter the respiratory tract of man and of numerous species of both wild and domestic mammals, and then change to the parasitic stage in which endosporulating spherules are formed. In the majority of the infections, the disease

is self-limiting and is manifested as a subclinical, localized pulmonary infection. In a small fraction of infected humans, 0.5%, pulmonary lesions develop along with a protean complex of clinical manifestations, extensive morbidity and a significant number of deaths. Even though a vast amount of the clinical symptomology is cataloged as common medical knowledge, the diagnosis is difficult and is, in effect, frequently missed. As a result, adequate preventative measures are often not undertaken.

Although it appears that there are adequate procedures available for the diagnosis of coccidioidomycosis, many problems exist. The primary disease is often confused with other acute pulmonary infections such as influenza, primary atypical pneumonia, bronchitis, bronchial pneumonia, or simply a common cold. Since this form of the disease most often resolves uneventfully, and without treatment, the patient is generally not compromised. Secondary coccidioidomycosis, however, requires therapy, and it is imperative that a specific diagnosis be rendered for a favorable prognosis. The disease must be differentiated from tuberculosis, neoplasia, other mycotic infections, syphilis, tularemia, glanders, and osteomyelitis of bacterial origin.

The choice method of diagnosis in coccidioidomycosis is by isolation of Coccidioides immitis from fluids or tissues taken from the body or by demonstrating typical

endosporulating spherules in body fluids or tissue sections. Unfortunately, the number of organisms seeded into the body fluids is minimal, and isolation of the organism is infrequent. Serologic methods are available, but they also have serious drawbacks. The most useful serologic test is the complement fixation reaction. Smith (119, 120), using the Kolmer method with two-hour binding of complement at 37 ^{o} C, concluded that over 80% of the patients with extensive disseminated disease produced a complement fixation titer of 1:16 or higher. With a single extra-pulmonary site of dissemination, however, only 15% of the patients were found to have a serum complement fixation titer of 1:16 or higher. Potentially fatal coccidioidal meningitis may occur with serum complement fixation titers less than 1:16 in 50% of the patients. Fewer than 10% of primary non-disseminating coccidioidomycosis patients examined had titers greater than 1:16. Limited coccidioidal disease, a chronic pulmonary cavity or solitary pulmonary granuloma, is usually accompanied by serum complement fixation titers of less than 1:16. In 60% of the cases with cavitary coccidioidomycosis, the patients have diagnostic fixation of complement (at least 4+ at 1:2 dilution of serum), but with solitary pulmonary granuloma, more than 70% of the patients had titers of 1:2 or less.

Sera from patients with various non-mycotic diseases have been reported by Schubert and Hampson (113) and

Kaufman and Clark (67) to fix complement in the presence of coccidioidal antigen. Thus, sera from patients with tuberculosis, pneumococcal pneumonia, staphylococcal empyema, central nervous system malacia, macular degeneration, pulmonary carcinoma, and regional enteritis fixed complement at titers of 1:2 to 1:8. Kaufman and Clark (67) also demonstrated reactions of coccidioidal antigen in immunodiffusion with sera from one patient with reticular cell sarcoma and one with central chorioretinitis.

The two best known endemic regions of coccidioidomycosis are the southwestern United States and northern Mexico. The area of endemicity within the United States include the states of Arizona, California, Texas, Nevada, New Mexico, and Utah. Northern Mexico contains two endemic areas which are ecologically comparable to the San Joaquin Valley of California. The first is termed the "Northern Zone" which is a continuation of endemic areas of the southwestern United States. This zone includes the states of Sonora, Chihuahua, Coahuila, Nuevo Leon, and Tamaulipas. The second zone of endemicity is the Pacific littoral zone. This encompasses the Sonora and Sinaloa states west of the Sierra Madre Occidental mountains. As delineated by Maddy (79), this area conforms to the ecological classification termed the "Lower Sonoran Life Zone." The zone is characterized by the presence of such plants as the creosote bush, mesquite, other cacti, yuccas, and agaves. Among the

animals found there are species of the pocket mouse, kangaroo rat, ground squirrel, long-eared desert fox, big-eared and white-haired bat, and a few birds including some owls. The climatic conditions of this region are semi-arid rather than completely dry; the average rainfall is 10 inches per year. Temperatures average 100° F in the summer then fall in winter to a mild $33\text{-}38^{\circ}$ F. It is estimated that 35,000 new human infections with Coccidioides immitis occur in California alone while the annual total for the entire endemic region is reported to be about 100,000 cases per year (3).

A recent study (43) compared the incidence of systemic mycoses requiring hospitalization during the years 1970 and 1976. The study reported the following facts concerning coccidioidomycosis: 1) the projected incidence of cases of coccidioidomycosis requiring hospitalization was 17.86 per one million population in the United States; 2) the average duration of hospitalization for persons with primary and secondary diagnoses of coccidioidomycosis was 11 to 12.2 days; 3) the projected cost in terms of diagnosis, hospitalization, and treatment for coccidioidomycosis was estimated to be 8.2×10^{6} dollars per year; 4) if coccidioidomycosis was the primary diagnosis, then 1.3% of the patients died; however, if the diagnosis was secondary to other disease states, the fatality rate was 4.4% per year; 5) the projected total number of deaths in the United

States in 1976 was 91. The percentage change in the crude mortality from 1970 to 1976 for coccidioidomycosis was 72.3%. The authors stated that the sharp increase in the incidence of coccidioidomycosis was due to the migration of susceptible individuals into the endemic area in the Southwest as well as the increasing rate of construction and other soil-disturbing activities which would lead to the raising of spore-bearing dust.

In some endemic areas, the positive skin test rate is 90% or more, the average being 50 to 70%. Generally, one-fifth of the positive reactors will develop an infection severe enough to cause temporary incapacity and to warrant medical care. Coccidioidomycosis is a problem, not because of the mortality it causes, but because of the morbidity associated with it. Even with this high rate of positive skin test reactions, the distribution of Coccidioides immitis in the endemic regions is usually sparse. It has been demonstrated that there are natural antagonists of Coccidioides immitis that inhabit the soil as well as adverse temperature and chemical factors which affect the survival of the organism in the soil (38, 44). Thus, it appears that Coccidioides immitis is rather poorly adapted to living in its accepted ecological niche. However, this assumption is directly contradicted by the fact that several outbreaks of coccidioidomycosis have occurred when soil containing arthroconidia became airborne either by a dust storm

or by the mechanical agitation of the soil in conjunction with an archeological dig (42, 133). Therefore, it must be that our methods of isolation for Coccidioides immitis from the soil within an endemic area are not yet adequate to unearth the true ecological niche of the organism.

Even though Coccidioides immitis is a pathogenic fungus, and it is probably the most virulent of all the etiologic agents of the human mycoses, serious disease is established in only about 0.5% of the population that is infected with C. immitis. In order to establish a definitive diagnosis of coccidioidomycosis, it is necessary to culture the fungus from clinical specimens, or demonstrate the characteristic endosporulating spherules in infected tissue. However, this is not always possible, and, in such cases, the patient's history and immunological tests can be used to establish a tentative diagnosis. The serological tests used routinely are complement fixation, immunodiffusion, latex particle agglutination, and skin reactivity (68). The routinely used antigen, coccidioidin, is prepared from the culture filtrate of the mycelial-arthroconidia phase of Coccidioides immitis. Recently, a new antigen, spherulin (75), was introduced, but it has not yet replaced coccidioidin as the antigen of choice in the various serological tests for coccidioidomycosis. Since both of these antigen preparations are crude cell extracts, not only is the antigenic content of these preparations unknown, but the

concentration of specific antigens may vary from one preparation to another. The variation in individual antibody responses makes it likely that crude extracts containing the widest possible range of antigen components will continue to be of practical value, although purified and standardized antigens are, of course, desirable objectives. Additionally, a detailed understanding of the structure of the antigenic component(s) should help to minimize the problem of cross-reactivity, and this would be made more satisfactory by precise immunochemical characterization of fungal antigens.

In order to produce a chemically homogeneous antigen with high specific activity, Ward et al. (132) used an alkali-soluble, water-soluble antigen (c-ASWS) extracted from the mycelial-arthroconidia phase of Coccidioides immitis. This antigen proved to be lacking in cross-reactivity when tested in histoplasmosis-infected animals and elicited a significant reaction in a dose one hundredth of the amount of a standardized coccidioidin dose (120). However, this antigen showed a certain amount of variability which was probably due to differences in the amounts of essential antigenic components in the different strains within the species. Subsequent analysis by two-dimensional immunoelectrophoresis (63) revealed that c-ASWS contained six antigen-antibody complexes in common with coccidioidin, spherulin, histoplasmin, and blastomycin. Therefore, a contradiction existed

since *in vivo* results with animal testing showed specificity without cross-reactivity, while *in vitro* two-dimensional immunoelectrophoresis analysis revealed antigen-antibody complexes in common with other fungal skin test reagents. This contradiction has not been fully explained. If results with histoplasmin (11, 80, 101) were indicative, it appeared reasonable to assume that further purification of the c-ASWS might result in an antigen which is highly specific for the serological diagnosis of coccidioidomycosis and is capable of being standardized.

As previously stated, a definitive diagnosis of coccidioidomycosis depends on isolation and identification of the fungus from clinical specimens. Emmons (39) showed that certain saprophytes, principally species of the genera *Auxarthron* and *Gymnoascus*, may resemble *C. immitis* and *vice versa*. Huppert *et al.* (59), in a study of 301 strains of *C. immitis*, noted a range of variations in colony characteristics and microscopic morphology which was so extreme as to indicate to the authors that many of the cultures might have been discarded as not being *C. immitis* even by experienced mycologists. They concluded that due to variation in *C. immitis* strains, it was necessary to meet two conditions to establish the identity of an isolate as *C. immitis*: 1) the production of tissue pathology in experimental animals; and 2) the production of endosporulating spherules in the tissues of such animals. Laboratories

which do not perform animal testing must submit cultures suspected of being C. immitis to reference laboratories for identification. In many instances, identification may take three weeks or longer. It can, in certain cases, be accomplished without the use of animals by converting the mycelial phase of C. immitis to the spherule phase (16, 21, 105, 129). However, these methods are not uniformly reliable and require the use of slide cultures which carry the risk of arthroconidia production and subsequent environmental contamination through aerosolization. Because of these problems, a promising new immunodiffusion technique, involving the analysis of specific cell-free antigens of C. immitis obtained by extraction of cells from agar slant cultures (69), has been developed. The test is specific and fairly rapid, but requires the use of reference antigens and antisera that are not commercially available. Thus, this technique is not available for use in many clinical microbiology laboratories.

An area not fully exploited for the diagnosis of coccidioidomycosis is the analysis of patient serum by gas-liquid chromatography (GLC). Although fairly well developed for use with bacteria (13, 28, 46, 82, 89, 99, 100), GLC for identification of fungal infections has been limited to infections due to Candida albicans (40, 48, 72, 86, 90, 91, 106, 140, 141). Sera from patients with candidiasis have been subjected to analysis by GLC to look for the presence

of D-arabinitol (40, 48, 72, 106, 140, 141) which has been shown to be a metabolite of Candida albicans (40, 48, 72, 106, 140, 141). Mannose, a sugar that is an integral part of the organism's cell wall, has also been looked for in patient's serum (90, 91). Additionally, serum has been subjected to silylation with the subsequent GLC analysis demonstrating chromatographic patterns of both carbohydrates and fatty acids that are unique to the patients infected with Candida albicans (86).

In light of the above, the following hypothesis will be will be tested: That C. immitis produces antigenic (species-specific) partially methylated 3-0-methylated heteromannans in the cell wall that can serve as a diagnostic marker of coccidioidomycosis. In order to test this hypothesis, this research will concentrate on the following three major objectives: 1) to develop a high performance liquid chromatographic (HPLC) methodology to separate and collect for analysis the various components of hetero-disperse fungal antigenic preparations; 2) to begin a chemical analysis of the alkali-soluble, water-soluble (c-ASWS) antigen preparation from Coccidioides immitis; 3) to develop a rapid GLC technique for the diagnosis of infection by C. immitis by looking for 3-0-methyl-mannose in serum from patients with coccidioidomycosis as this methylated sugar is found in the cell walls of C. immitis and not in the walls of other disease-causing fungi.

CHAPTER II

REVIEW OF THE LITERATURE

History

As with many of the mycoses, the discoverers of coccidioidomycosis initially described the organism as a protozoan. Posadas (103) published the preliminary reports of a case in Argentina while in the same year, 1892, Wernicke (134) described the same patient in a paper published in Germany. At almost the same time that the disease was described in Argentina and Germany, Rixford and Gilchrist were studying a case of coccidioidomycosis in California (104). Rixford and Gilchrist (104) named the organism, which they saw in clinical material, Coccidioides (coccidia like) immitis (im = not, mitis = mild) and described it as a protozoan of the class Sporozoa. The true nature of the etiological agent of coccidioidomycosis was finally elucidated by Ophuls and Moffitt (94). In 1905, Ophuls (95) published his works on the life cycle of C. immitis. From this point forward, the study of coccidioidomycosis became associated with the state of California. In 1915, Dixon (34) reviewed the forty known cases of the disease and stressed the importance of its occurrence in the southern California area. In 1932, Stewart and Meyer

(126) isolated C. immitis from soil in the San Joaquin Valley and thus, established the soil as a reservoir for the organism in that area. In 1938, Dixon and Gifford (35) named the disease coccidioidomycosis and delineated the primary and secondary phases of the disease process. Following this report, C. E. Smith began an extensive study of coccidioidomycosis in the San Joaquin Valley. He gathered data concerning the incidence, severity, and epidemiology of the disease (115, 117, 119) and developed a precipitin test and a standardized coccidioidin skin test (117, 119). Studies and reviews of the clinical and pathologic forms of the disease, its epidemiology, ecology, serology, and therapy have made it one of the best described of the mycotic infections (2, 4, 5, 37, 125).

Development of an Effective Skin Test Antigen

Coccidioidin, an extract obtained from cultures of Coccidioides immitis, is the principal antigenic reagent employed in intradermal and serological tests for coccidioidomycosis (120). Early clinical interpretations, however, had been complicated by evidence of cross-reactivity in serum specimens obtained from patients with diseases caused by different fungi (17, 18, 118).

The evolution of the skin test reagent as a diagnostic tool has included the use of antigens prepared by a variety of procedures. Cooke (23, 24), while working with patients

who had coccidioidal granuloma, used a bouillon culture filtrate, a thick suspension of powdered mycelia, and a suspension of spherules obtained from pus as test extracts. These extracts were given both intradermally and by scarification. He obtained a pustular reaction not only in a patient with coccidioidal disease, but also in six control subjects. The results led Cooke to conclude that there was no specific skin test reaction in coccidioidal granulomas. An antigenic preparation was also obtained from broth culture filtrates by Cummins and Saunders (29). Using this preparation, they could not demonstrate agglutinins, precipitins, or complement fixing substances in the serum of patients and animals with coccidioidal granuloma. They further reported that the concentrated filtrate from heated and unheated broth cultures caused a cutaneous reaction in some of their infected animals, and to a lesser degree, in uninfected rabbits. The reaction was seen to be the most pronounced when the unheated filtrate was used as the antigen. Davis (31) did not detect agglutinins in the serum of a patient he studied, but attained complement fixation reactions when a concentrated culture preparation was employed as the antigen. An intradermal skin reaction occurred with a preparation of killed, homologous organisms that was more intense than the reaction observed following the injection of controls consisting of 1) a suspension of pure agar, 2) a suspension of ground Sporothrix

schenckii, and 3) a suspension of ground Blastomyces dermatitidis. Hirsch and Benson (52) and Hirsch and D'Andrea (53, 54) prepared a skin test reagent from a filtrate of cultures grown in peptone broth with added placenta tissue extract, 0.1% dextrose and 0.2% dibasic sodium phosphate. Similar reactions were obtained with cells grown in an ammonium lactate synthetic medium containing 0.5% sodium chloride. From these data, they concluded that the growth of C. immitis in liquid media liberated a "soluble specific substance" which caused skin reactions in both C. immitis infected animals and in patients with coccidioidal granuloma like those occurring when tuberculin is injected into animals with active tuberculosis.

Jacobson (65) used a culture filtrate of Coccidioides immitis grown in Sabouraud's dextrose broth to skin test six healthy individuals. In all six cases, Jacobson's coccidioidin did not elicit a positive response. However, in one patient with blastomycosis, a positive skin test reaction occurred upon testing with this preparation. Six additional patients with coccidioidal granuloma also reacted positively to skin testing with this preparation. Jacobson (65) felt that these data indicated that C. immitis produced a filterable substance that gave a characteristic skin test reaction in patients with coccidioidal granuloma which mimicked the reaction of tuberculosis patients to intradermal tuberculin. Hurwitz and his colleagues (64) studied a group

of 449 patients in which a subset of 12 patients had coccidioidal granuloma. Another subset of 177 patients had tuberculosis, and the remaining 260 patients had miscellaneous diseases. Skin testing of these patients with an antigen derived from a culture of filtrate of C. immitis revealed the following: 12 of 12 patients with the coccidioidal granuloma gave a positive skin test; 49 of 177 or 27.5% of those with tuberculosis gave a positive skin test with the antigen; and finally, 11 of 260 or 4.2% of patients with miscellaneous diseases reacted to skin testing with the C. immitis derived antigen. This study was the first to raise the question of the possible cross-reactivity of a skin test preparation extracted from C. immitis with tuberculous patients.

Kessel (70, 71) produced a skin test preparation by growing six strains of C. immitis in a 2% dextrose veal infusion broth and incubating at 37° C for a period of six weeks. The cultures were then autoclaved at 121° C for fifteen minutes, and the cell-free broth was discarded. The fungal growth was harvested, ground using sterile sand and resuspended in the original medium. The resulting mixture was shaken, decanted to exclude the sand, and finally, filtered through Seitz filters. The filtrate was diluted 1:10 with normal saline, bottled and tested for sterility. Twenty-six individuals with proven coccidioidomycosis were skin tested with this preparation, and 22 gave positive

reactions. The four patients who gave negative reactions were tested only one week prior to death and could have been in a state of anergy. Of 228 tuberculosis patients tested with the same antigen, 191 gave negative responses while 37 gave positive skin responses. Of the 37 patients that responded positively to skin testing with the C. immitis antigen, 26 were laborers in the San Joaquin Valley of California and 7 were from New Mexico and Arizona. Thus, in 33 of the 37 positive responders among the tuberculosis patients, a correlation was drawn between probable exposure to C. immitis and the positive reaction upon skin testing with the C. immitis derived antigen. The remaining 4 responders had no apparent prior exposure to C. immitis. Beck, Traum, and Harrington (10) produced positive intracutaneous reactions in infected cattle by using an autoclaved beef broth culture filtrate of Coccidioides immitis that was concentrated to half volume by evaporation using a water bath. Giltner (47) obtained negative subcutaneous tests in animals with preparations of C. immitis made in a manner similar to the way in which tuberculin was produced (114). Stewart (127) prepared coccidioidin from mycelia harvested from one-year old ammonium chloride-sodium acetate liquid synthetic medium cultures which were ground by ball mill. Purification was accomplished through the use of colloidin filters. This preparation was standardized using infected, sensitized guinea pigs. In 1947, Smith (115) and

Smith et al. (117) prepared the coccidioidin against which all coccidioidin preparations are now standardized. Originally, Smith prepared coccidioidin by using the ammonium lactate synthetic medium suggested by Hirsch and Benson (52), and Hirsch and D'Andrea (53, 54) as well as the ammonium chloride-sodium acetate medium used by Stewart and Meyer (126). However, Smith was unable to obtain coccidioidins with sufficient potency within a reasonable period of time. He finally succeeded in preparing an antigen by growing four strains of C. immitis in a pancreatic digest of casein peptic digest-glucose broth for five weeks, adding merthiolate to a final concentration of 1:10,000 and then filtering through Seitz filters. The coccidioidin produced was found to be satisfactory when used in epidemiological studies. However, the preparation contained too much foreign protein. Since the asparagine synthetic medium used for the production of tuberculin had been proven to be non-antigenic when tested alone (114), Smith decided to use it for the production of coccidioidin. By reducing the glycerine content to 2.5% and substituting part (0.7%) of the asparagine with ammonium chloride, a satisfactory coccidioidin was prepared. This coccidioidin was used for extensive serologic testing (120) and then, became the standard skin test reagent. Accordingly, Smith's method of coccidioidin production, by growing the fungus in a modification of the asparagine, salts, and

glucose medium used for the preparation of PPD tuberculin, became the standard method for coccidioidin production. Even with the standardized method of production, there remained the problem of irregularity in the appearance of satisfactory amounts of skin test antigens in the culture filtrates (96). Thus, many lots of coccidioidin are discarded because of a lack of specificity and potency. This problem has yet to be solved so that lots of coccidioidin are produced which have a consistent level of potency from batch to batch.

Because coccidioidomycosis in man is characterized by the presence of spherules in tissue and endospores, and since coccidioidin is prepared from the mycelial phase of C. immitis, Levine and his co-workers in 1969 (75) suggested that a skin test antigen prepared from spherules might be more effective in identifying persons with prior exposure to C. immitis. Subsequent reports by Levine and Stevens have borne out this observation in animals (75), men, women, and children in regions (76, 77, 123) endemic for coccidioidomycosis and in patient populations with documented clinical disease (9, 124). These studies showed that the use of spherulin can lead to the detection of approximately one-third more persons who have been sensitized to C. immitis than does the use of coccidioidin. Spherulin is produced by suspending isolated spherules and endospores in distilled water and then incubating this mixture with shaking at 35 ^{o} C for 20 days. After the incubation period, the cells were

sonicated (20,000 cycles per second) at 0-3 ^{o} C for one hour. The resultant mixture was centrifuged to remove whole cells and cellular debris, and the supernatant fluid was decanted and filtered. The sodium chloride content of the supernatant was then adjusted to 0.85% and 1:10,000 merthiolate was added (75, 123) as preservative.

Recently, in efforts to produce more effective antigens for use in testing the immune response in coccidioidomycosis, Ward et al. (132) produced an alkali-soluble, water-soluble fraction (c-ASWS) from the mycelial-arthroconidia phase of C. immitis. Cell walls obtained from a strain of C. immitis designated 46 were extracted with sodium hydroxide, and the resulting solution was dialyzed against distilled water and lyophilized. This alkali-soluble, water-soluble fraction extracted from C. immitis mycelium and arthroconidia was designated c-ASWS. When tested against Smith's coccidioidin, it produced skin test reactions in a greater proportion of the guinea pigs used (92 vs. 54%), and it gave a response in a dose only one-hundredth as strong as that required for Smith's coccidioidin. The c-ASWS was also found to not cross-react in guinea pigs sensitized to Histoplasma capsulatum antigens. In other testing (26), c-ASWS has been used to demonstrate that the cellular immune defect, in terms of antigen recognition, that occurs in patients with progressive, disseminated coccidioidomycosis, is specific for C. immitis.

Since coccidioidin failed to elicit a positive skin test reaction in some patients known to have coccidioidomycosis (120), the possibility that different strains of C. immitis produced different amounts of antigenic components was considered. It is possible that a particular strain of C. immitis could produce antigenic components different from those of other strains and that this difference could lead to false negative tests. Stewart (127) observed patients with proven cases of coccidioidal granuloma who reportedly failed to give positive skin test reactions with standard preparations of coccidioidin. To test the possibility of antigenic variability among strains of C. immitis, guinea pigs were infected with different strains of C. immitis and antigens were prepared from these different strains. All the test animals died with the typical lesions of coccidioidal granuloma. Positive skin test reactions were only obtained by using antigen preparations obtained from the strain used to infect the test animals. These data suggested the need to use polyvalent antigen preparations. Stewart and Kimura (128) also suggested that there may be more than one immunologic strain of C. immitis. Kessel's (71) skin test preparation was prepared as a polyvalent mixture of antigens from five strains of C. immitis to provide broader antigenic coverage. Guinea pigs infected with the five strains of C. immitis used in the preparation of this skin test preparation were given skin tests with the respective homologous and

heterologous antigens prepared separately from each strain of the fungus. All the animals tested gave positive skin test reactions with both homologous and heterologous antigens. In testing fourteen patients with clinically proven coccidioidomycosis, Denenholz and Cheney (32) found no patient who did not react to skin testing with the standard coccidioidin. They suggested that the standard coccidioidin was able to elicit skin test reactions in patients with infections caused by different strains of C. immitis.

Smith and associates (117), in order to provide maximal antigenic coverage, also used multiple strains of C. immitis to produce coccidioidin. The first coccidioidin produced was made from four strains of C. immitis and later coccidioidins were produced which utilized ten strains of C. immitis for their production. The ten strains included C. immitis isolated from patients with disseminated infections, pleural effusion, uncomplicated coccidioidal infections, cavitary pulmonary disease, and strains which had been isolated throughout the endemic southwest region of the United States. To further investigate possible strain differences, whenever feasible, Smith et al. (117) made an autogeneous coccidioidin for clinical trials with any patient who failed to react to their standard coccidioidin. However, the autogeneous coccidioidins were not shown to be more effective in eliciting a reaction than the standard coccidioidin.

Therefore, the possibility of antigenic strain variation, while not eliminated, was thought to be a rare occurrence.

Four strains of C. immitis were tested by Scalarone et al. (109) for spherulin production. All the strains tested produced spherulin that elicited a skin test reaction in infected guinea pigs that was equivalent to the reaction obtained when the infected animals were skin tested with the spherulin produced from C. immitis strain 46. In testing c-ASWS antigen preparations produced from three different C. immitis strains in Coccidioides and Histoplasma sensitized guinea pigs, Ward et al. (132) found no significant difference in activity among the three c-ASWS preparations. All three preparations produced reactions in 60% of the homologous and heterologous sensitized animals. Furthermore, none of the preparations caused reactions in the Histoplasma sensitized animals. Kaufman (68) has indicated that unless coccidioidin derived from multiple strains showed a greater biologic activity, as compared to coccidioidin prepared from single strains, there was no need to produce multiple strain skin test reagents.

Rowe et al. (107, 108) studied soluble antigens from the Silveira strain of C. immitis by immunodiffusion. In the first study (107), the effects of different cultural conditions on the development of antigens were studied. The authors concluded the following: 1) the same amount of antigenic material was produced when cultures were incubated

at 30 °C or at 37 °C; 2) as the amount of glucose in the medium was increased, there was an increase in the amount of antigenic material produced; 3) with increasing periods of incubation, there was a concomitant increase in the amount of antigenic material produced; and 4) extraction of the washed, isolated mycelial mat with ethylene glycol released an antigen that was always present. In the second study (108), a number of different techniques were used in the preparation of antigens for examination by immunodiffusion. When the different antigen preparations were tested against antiserum from a patient with culturally proven coccidioidomycosis, six precipitin bands were visible. The following conclusions were drawn from this study: 1) the antigens produced were polysaccharides or glycoproteins; 2) different preparation techniques yielded different amounts of antigen as shown by the clarity of the six precipitin bands; and 3) exposure of the antigenic preparations to either extremes of temperature (≥ 60 °C for one hour) or pH extremes (≤ 4.0) decreased the number of precipitin lines seen on analysis by immunodiffusion. When analyzed together, the results from the two studies (107, 108) indicated that differences in growth rate, cell wall permeability, or extraction technique could explain the various results obtained with different skin test reagents rather than a difference due to strain variability.

Landay et al. (73) produced hyperimmune sera in rabbits against spherules and arthroconidia of C. immitis. The antibody content of these sera was studied by the agar gel diffusion method. Anti-spherule pooled serum formed multiple precipitin bands with extracts of both spherules and arthroconidia. However, the anti-arthroconidia pooled serum formed a single precipitin band with arthroconidial extracts and none with the spherule extracts. The spherule extract formed precipitin bands only with anti-spherule sera. Arthroconidia extracts formed precipitins with anti-arthroconidia, anti-spherule, and anti-Histoplasma capsulatum sera. Based upon these data, the authors concluded that extracts from the spherule phase of C. immitis differed antigenically from those extracts obtained from arthroconidia.

In a study of several strains of C. immitis, Gale et al. (45) observed a strain variance in the production of components toxic to mice as well as differences in the ability to invade, multiply, persist, and disseminate the host. Huppert and associates (60) immunized mice with a formalin-killed endosporulating spherule preparation from C. immitis strain Silveira and then challenged the mice intranasally with arthrospores from seven heterologous strains of C. immitis. Two of the strains tested were culturally and morphologically typical of the species while the remaining five strains were atypical. The immunized animals were well protected against challenge doses that were

lethal to a majority of the control animals regardless of the strain of C. immitis that was used for the challenge. The authors suggested that these seven strains were at least immunologically similar, although not necessarily identical, and that a vaccine prepared from a single strain of C. immitis would be practical for a program of immunization.

Analysis of the Antigenic Components

The nature of the antigenic component(s) has not yet been determined. Hirsch and Benson (52) reported that in liquid medium, C. immitis liberated a soluble substance which, in infected animals and in patients with coccidioidal granuloma, caused a skin reaction analogous to the tuberculin reaction. This soluble substance was not destroyed by heating to 80 ^{o}C for 30 minutes. Hirsch and D'Andrea (53, 54) precipitated the active substance from culture media with alcohol and obtained a white precipitate that was found to be soluble in 0.85% sodium chloride, dilute acid, and alkali solutions. Analysis revealed that the precipitate was composed of 3-4% nitrogen, and contained 20-40% reducing sugar measured as glucose. The osazone prepared from a hydrolyzed solution resembled the osazone formed with dextrose. This would indicate that the sugar was either dextrose or its epimer mannose. Stewart and Kimura (128) found that 1.0 ml of undiluted coccidioidin contained 3.89 mg of total nitrogen--0.97 mg as protein nitrogen and 2.92 mg of

non-protein nitrogen. The active substance was characterized as a heat stable, dialyzable compound that was probably polysaccharide in nature. Hassid et al. (51) also studied a specific soluble substance from C. immitis that was isolated from a culture filtrate. This substance was soluble in water. It contained 3.23% total nitrogen of which only 0.6% was associated with amino acids. No acetyl groups were present in this soluble substance. A reducing sugar value of 9.5% was measured as glucose. Upon hydrolysis, the carbohydrate portion was found to contain galacturonic acid, glucose, and an unknown sugar (1:6:3). Pappagianis et al. (97) studied a soluble polysaccharide isolated from culture filtrates of C. immitis and found that mannose was the main carbohydrate present. Chromatographic examination of acid hydrolysates revealed that the polysaccharide also contained small amounts of both galactose and an unidentified carbohydrate monomer. The crude polysaccharide preparation contained 3-4% nitrogen which correlated to a peptide content of 15-20%. Attempts to separate protein and carbohydrate moieties were unsuccessful. However, treatment of the crude antigen with either 40% sodium hydroxide (room temperature) or hot 30% potassium hydroxide resulted in inactivation of the antigen. A molecular weight of 31,700 was determined by osmometry. Use of the analytical ultracentrifuge revealed a single component peak with a value of 2.2 Svedberg units. Thus, the molecular weights obtained by osmometry and

analytical ultracentrifugation were not incompatible with one another. Immunodiffusion showed multiple precipitin bands which pointed toward heterogeneity. These studies were corroborated by Pappagianis et al. (98) when they studied antigens from three-day-old mycelial cultures of C. immitis. Again, the antigen that was isolated was found to contain 14-18% nitrogenous compounds and 60-70% carbohydrate as mannose. This time they were also able to separate the fraction containing the complement fixing activity from the fraction exhibiting skin test activity. McNall et al. (84) examined a polysaccharide antigen obtained from C. immitis mycelial cell walls. Hydrolysis of the antigen and analysis of the hydrolysate by thin layer chromatography revealed the presence of four sugars: glucose, mannose, glucosamine, and galacturonic acid (3:2:3:1). The authors, using tests to determine the degree of polymerization, calculated that there were 400 polysaccharide residues per antigen molecule.

Marcus et al. (83) isolated both protein and polysaccharide components from coccidioidin. Pronase and trypsin treatment of the two fractions depressed their skin test reactivity. The polysaccharide component was separated into six fractions by chromatography on a column of DEAE-cellulose. The six fractions elicited different amounts of response when used as skin test antigens. Treatment with proteolytic enzymes also depressed the skin test reactivity of the six polysaccharide fractions.

Anderson, Wheat, and Conant (7) studied the following two antigenic preparations: 1) the mycelial culture filtrate from an actively growing C. immitis culture; and 2) a toluene autolysate obtained from the mycelia of this actively growing broth culture. Both the crude culture filtrate and the toluene autolysate were subjected to exhaustive dialysis against multiple changes of distilled water. The dialysates of the two antigenic preparations were negative for skin test activity. All of the activity was contained in the retentate. Mannose was found to be the main hexose in both preparations while 3-0-methyl mannose was also present. The retentate was separated into two components by gel permeation chromatography on Sephadex G-25. The skin test active substances fell into a molecular weight range of 10,000-60,000 daltons. Preliminary disc gel electrophoresis studies revealed three protein bands from both culture filtrates and toluene autolysates. The authors concluded that skin test reactivity correlated with a heterodisperse mixture of antigens rather than with a single, discrete antigen.

Standardization of Skin Test Antigens from *Coccidioides immitis*

At present, there is no standardized method for determining the potency of various coccidioidins for use as skin test reagents. Smith et al. (117) have observed as much as a five-fold difference in potency in batches of coccidioidin

even when the same multiple strain inoculum was made in an identical manner, incubated in the same incubator, and placed in the same spot in the incubator. Stewart and Kimura (128) recognized the need for standardization procedures for coccidioidin production. Their solution to the standardization procedure was to define one skin test unit as the smallest amount of reactive substance contained in 0.1 ml of solution that will, on the average, in sensitized guinea pigs, provoke in 24 hours an erythema of 1.0 cm in diameter persisting for 24 hours. Through testing various dilutions of coccidioidin on sensitized guinea pigs and human subjects, they found that one skin test unit was contained in 0.1 ml of a 1:100 dilution of coccidioidin. New preparations of coccidioidin would then go through the dilution protocol to determine the optimal dilution that contained one skin test unit. The use of 10-100 skin test units was recommended for routine testing.

Smith et al. (117) found that guinea pigs were unsuitable for use as test animals in standardizing coccidioidin preparations since C. immitis infected animals varied greatly in their response to coccidioidin as a skin test antigen. For these reasons, they standardized new lots of coccidioidin by making serial dilutions and testing the new coccidioidin against the old coccidioidin in three or four individuals who were known to be sensitized to C. immitis. They stressed that the difference in the

non-specific reaction of the various lots of coccidioidin made it very important to check each lot in human subjects. Sutton and Marcus (130) developed a bioassay that yielded a potency ratio with known confidence limits for new lots of coccidioidin relative to a standard lot. The potency ratio was derived after performance of an analysis of variance on the data. The potency ratio could then be used as a dilution factor to standardize new lots of coccidioidin. This method was fairly reproducible but did not work on coccidioidins that demonstrated a lack of parallelism of the standard and test log-dose responses. Wallraff and Wilson (131) suggested a standardization procedure that involved plotting a dose response curve based on the amount of non-dialyzable nitrogen and the size of the skin test reaction. This procedure has yet to come into common usage.

Huppert et al. (62) proposed using two-dimensional immunoelectrophoresis as a tool to analyze and compare new lots of coccidioidin. Two-dimensional immunoelectrophoresis revealed that coccidioidin contained 26 antigen-antibody precipitates while spherulin contained only 12 antigen-antibody precipitates. Among these precipitates, 10 were common to both preparations, 16 were unique to coccidioidin, and 2 were unique to spherulin. Since the area under an antigen-antibody precipitate is related to the quantity of antigen-antibody present, establishing a reference with fixed amounts of antigen and antibody would enable evaluation of

successive batches of coccidioidin for both qualitative and relative quantitative content of individual antigens as compared to a reference standard. Theoretically, the method would be an excellent tool for standardization of antigenic preparations used as diagnostic aids in immunologic testing.

Specificity of Skin Test Antigens

The specificity of coccidioidin and other skin test antigens has been widely discussed. One of the early questions (10) was that of possible cross-reaction with tuberculin. Beck et al. (10) reported that three calves that had reacted to intradermal tuberculin prior to death had actually died of coccidioidal granuloma. To further study a possible cross-reaction, they produced coccidioidal granulomas in 59 guinea pigs, and then, tested these animals for reaction to intradermal tuberculin. It was found that a few of the guinea pigs gave weak reactions to intradermal tuberculin. The frequency of coccidioidal sensitivity in the general population, especially in tuberculosis cases, made the Hurwitz group (64) question the specificity of coccidioidin. However, since the coccidioidin they used was the equivalent of a 1:10 dilution, it might well have had some non-specific components associated with it. More importantly, residence histories were not taken, and the long duration of prior sensitivity to C. immitis was not yet appreciated. Kessel's series (70, 71), taking into account residence, bore

out the specificity of their coccidioidin with respect to tuberculous humans and his careful experimental studies in guinea pigs gave additional evidence against cross-reactions with tuberculosis. However, Aronson et al. (8) provided the clinching proof that there was no cross-reactivity of coccidioidin in the tuberculous patient. Among the Alaska Indians, in whom 96% reacted to tuberculin, none reacted to a 1:100 coccidioidin, and only three (1.1%) reacted to a 1:10 coccidioidin preparation. In South Dakota Indians with no reaction to coccidioidin, two-thirds reacted to tuberculin and heteroallergenic reactions were demonstrated to the proteins of Mycobacterium smegmatis, Mycobacterium ranii, and Mycobacterium marinum. Finally, Arizona Indians in the northern part of the state, where 84% were tuberculin positive, were only 15% coccidioidin reactive, and with the reactors, a history of exposure in the highly endemic southern Arizona area could be demonstrated.

Smith et al. (118) performed simultaneous coccidioidin and histoplasmin skin tests on both normal, healthy military personnel and on those with coccidioidomycosis. Among the healthy population that was non-reactive with coccidioidin, 26% reacted positively to histoplasmin. A number of the personnel that reacted to coccidioidin had had no known opportunity of acquiring coccidioidomycosis. All of these reactors were also reactive to histoplasmin. In fact, the stronger the histoplasmin sensitivity, the more frequent and

larger the non-specific reaction to coccidioidin. In the group of military personnel that had coccidioidomycosis, they showed non-specific cross-reactions to histoplasmin. Again, the greater the reaction to coccidioidin, the greater was the non-specific reaction to histoplasmin.

Huppert et al. (63) studied fungal antigens used in skin testing and serological tests by the use of two-dimensional immunoelectrophoresis. Results showed that of the 26 antigen-antibody precipitates in the coccidioidin reference system (62), 10 were found in common with an antigenic extract derived from Histoplasma capsulatum, and 12 were found in common with an antigenic extract derived from Blastomyces dermatitidis. Precipitates numbered 18, 19, 20, 23, 25, and 26 were found most frequently in all the preparations tested. Cox (27), using the lymphocyte transformation assay, demonstrated cross-reactions among antigens derived from C. immitis, B. dermatitidis, and H. capsulatum. All the preparations tested elicited cross-reactivity in this test. Cox stated that until new chemical or immunochemical procedures or both are employed to fractionate the antigens of C. immitis, H. capsulatum, and B. dermatitidis, cross-reactivity will continue to diminish the efficacy of these antigens. Further, until the antigens of the three fungi are fractionated and characterized, the likelihood that lymphocyte transformation assays will prove useful in distinguishing clinical infection between these three systemic fungi is doubtful.

Thus, coccidioidin is probably a heterodisperse mixture of antigens whose reactivity does not lie within a specific, distinct fraction. More work needs to be done before antigens can be prepared that are species specific and useful for testing serological and cell mediated immune responses in order to correlate the hosts' immunological responses with the diagnosis, course, and prognosis of the clinical disease.

Diagnosis of Coccidioidomycosis

The clinical picture presented by coccidioidomycosis is so protean that it must be differentiated from acute pulmonary infections, most chronic infectious diseases, and some of the malignancies. The most direct way to diagnose coccidioidomycosis is to isolate the etiological agent or to demonstrate typical endosporulating spherules in a direct examination of sputum, pus, pleural fluid, or other body secretions. Biopsies can be examined by both hematoxylin and eosin and/or silver strains, and an adequate identification of C. immitis in tissue can be made. Every effort should be made to recover the organism from infected tissue through prudent use of bronchoscopy, transtracheal biopsy, and/or other aspiration and biopsy techniques. However, when these measures are considered to be too invasive, immunodiagnosis can sometimes provide an alternative way of making the diagnosis.

Coccidioides immitis is not a fastidious organism. It grows readily on all commonly used laboratory media. On artificial media, the fungus produces an abundant aerial mycelium that is initially white, but usually becomes tan to brown with age. As the culture matures, thick-walled arthroconidia develop that alternate with thin-walled empty cells (disjunctures). The arthroconidia are barrel shaped, 2.5 to 4 by 3 to 6 μm in size, and are released by fragmentation of the mycelium. The arthroconidia can be released by the slightest disturbance and become airborne. As few as 10 arthroconidia are able to produce a pulmonary infection in primates (22).

Laboratory identification of C. immitis is hazardous, and over 200 cases of laboratory-acquired coccidioidomycosis have been reported (41, 50, 66). Thus, the mishandling of any unidentified culture of a white mold can lead to a case of laboratory-acquired coccidioidomycosis. The identification of C. immitis, once isolated, depends upon certain described colonial and sporulation patterns. However, it has been shown that isolates of C. immitis exhibit a wide range of both colonial characteristics and microscopic morphology such that many clinical isolates have been discarded by various laboratories as not being C. immitis (59). In some labs, the identification of atypical C. immitis isolates happens only because all mold isolates are routinely identified. Evidence (59) indicates that as

many as 25% of the laboratory isolated strains of C. immitis are discarded as being merely contaminants. Further studies (40) have shown that arthroconidia which bear slight or close resemblance to those of C. immitis are found in the haploid forms of many basidiomycetes, in species of Oospora, Oidiodendron, Coprotrichum, Coremiella, and several genera of the Gymnoascaceae. The arthroconidia of Auxarthium umbrinium bear a closer resemblance to the arthroconidia of C. immitis than most of the other non-pathogenic fungi. Since this fungus is found in many areas of the world, it can be isolated and misidentified as C. immitis. Until this potential for coccidioidal atypicality is fully understood, the careless handling of clinical fungal isolates can result in the aerosolization of potentially infective arthroconidia from unidentified cultures of C. immitis. The only acceptable proof for the identification of C. immitis has been inoculation of laboratory animals with the concomitant establishment of disease in which endosporulating spherules can be demonstrated in tissue sections. Saprophytic fungi are unable to produce disease in healthy laboratory animals. However, most laboratories lack the proper facilities for handling the animals once they are infected and must, therefore, submit suspected C. immitis isolates to reference laboratories for identification. Definitive identification can take three weeks or longer. To avoid the use of infected laboratory animals, techniques have been developed

that allow the laboratory to convert the mycelial phase of C. immitis into the tissue phase, (spherules and endospores) in vitro (16, 21, 105, 129). These methods lack reliability or make use of slide cultures which carry the risk of aerosolization of infective arthroconidia in the laboratory. A new and extremely promising technique uses immunodiffusion to analyze an extract taken directly from the fungal culture (122). The specificity of the test is based upon the fact that C. immitis produces antigens that are not produced by other morphologically similar fungi. This method has been tried by three independent laboratories of various sizes and found to be 100% reliable in the identification of even atypical C. immitis cultures (36). However, this method requires having both reference antigens and antisera that are not commercially available.

Fungal infections are difficult to diagnose from two standpoints: 1) over 60% of all patients that have a primary pulmonary infection are asymptomatic, and 2) even in disseminated fungal disease, blood cultures remain negative. The following serological tests are available for use in the diagnosis of coccidioidomycosis: the tube precipitin test (TP), latex particle agglutination test (LPA), complement fixation (CF), immunodiffusion (ID), and counter-immunoelectrophoresis (CIE).

The tube precipitin test usually becomes positive by the first week of clinical illness, but the frequency of

positive results declines to less than 10% by the fourth month. However, in patients who develop infection in the absence of any symptoms, the tube precipitin test may never become positive. In patients with mild disease, a positive tube precipitin test may not be followed by a positive complement fixation (IgG) antibody response. The TP test is not quantitative and is usually read as positive or negative. The test is of no value in the study of spinal fluid from patients with suspected meningitis caused by C. immitis.

The latex particle agglutination test presumably measures the same IgM antibody response as does the tube precipitin test, but it is more sensitive and more convenient. While much easier to perform, the latex particle agglutination test is limited by the fact that it has a false-positive rate of between 6-10% in patients with non-coccidioidal disease (20, 60). It is also possible that patients in later stages of infection may not produce a sufficient quantity of IgM antibody to be detected by this test, and so may give a false-negative test result. Finally, the latex particle agglutination test is of value in detecting antibody in the cerebral spinal fluid, but, in fact, gives false-positive results with this fluid (99).

The complement fixation test becomes positive early and tends to remain positive for long periods of time. Therefore, a single complement fixation test may indicate early reactive disease or a residual positive from a

previous infection. Properly performed, the complement fixation test can be a valuable guide to diagnosis (conversion from negative to positive, or documentation of a four-fold rise in titer during a current illness), and especially to prognosis (116, 119). High or rapidly increasing complement fixation titers are generally associated with a poor prognosis because they usually reflect the presence of extrapulmonary dissemination (116, 119). A significant decrease in the complement fixation titer during therapy is considered an indication of improving status of the patient (116, 119). However, the complement fixation test is costly, time-consuming, and requires a reference laboratory for its reliable performance. Additionally, the complement fixation titers obtained are not readily comparable from laboratory to laboratory (61).

The immunodiffusion test was developed by Huppert (55, 56, 57) to overcome the technical difficulties associated with the complement fixation test, and thus enabled non-reference laboratories to screen patient samples for coccidioidomycosis. Through the use of reference antigens and antisera, the behavior of the precipitin lines, as they intersect, provides important information with regard to the identity or lack, therefore, of the antigen/antibody systems. Complete development of the test requires 24 to 72 hours and is not quantitative as generally run. However, placement of serially diluted serums in the peripheral wells

converts the test to one in which titers can be measured. The immunodiffusion test is nearly equal in sensitivity to the complement fixation test when employed with positive coccidioidomycosis sera, but often results in the determination of lower titers (56).

Counterimmunoelectrophoresis provides a method for the rapid movement of antigen and antibody into proximity so that antigen-antibody complexes can form and precipitate. However, the counterimmunoelectrophoresis test sacrifices the ability to demonstrate a relationship between antibodies in patient's sera with those contained in reference sera. At present, its principal advantage is speed. Counterimmunoelectrophoresis has been shown to be as sensitive as complement fixation and double immunodiffusion (98 and 100% correlation, respectively) and as specific as double immunodiffusion (1). The counterimmunoelectrophoresis titers were found to be lower than those found with the standard complement fixation test. This test has not yet found widespread acceptance for use in the mycological laboratory.

An additional problem is encountered when working with fluids from immunosuppressed patients; antibody titers may be extremely low or lacking in patients on immunosuppressive therapy or who have immunologic disorders that preclude normal immunoglobulin synthesis (33, 112, 121). Studies (26) have also demonstrated that patients with progressive coccidioidomycosis have a cellular immune defect, that is,

in terms of antigen recognition, specific for C. immitis. Thus, in these patients or in patients who are immunosuppressed, tests that evaluate the cellular immune status of an individual are not of value in diagnosing an ongoing infection caused by C. immitis.

It should be obvious from the preceding discussion that other methodologies must be developed in order to be able to diagnose coccidioidomycosis with greater speed and accuracy. One method that has shown potential in other disease processes has been gas-liquid chromatography. The main advantage of this technique is its use of body fluids which can lead to a rapid diagnosis.

The analysis of body fluids is very complex. Since microorganisms make thousands of compounds, the search in vivo has been limited to examining the numbers and types of compounds that fall within a given chemical class. Carboxylic acids, hydroxy acids, alcohols, amines, and nitrosamines have been extensively studied in bacteria while neutral sugars, lipopolysaccharides, lipids, and long chain fatty acids have been studied in bacteria and fungi and viruses. Currently, it is not routinely possible to assay body fluids by GLC for the diagnosis of infection caused by bacteria, fungi, or viruses.

Mitruka et al. (87) showed that GLC can be used to detect viral infections in canines. The authors were able to show that viral infections could be detected by recognizable

GLC patterns of metabolic products produced both *in vivo* and *in vitro*. Even though the authors were able to show that detection of viral infections was feasible, they felt that the methodology was not reliable enough to be useful in the clinical laboratory. Cherry and Moss (19) wrote an editorial in 1969 discussing the possible role that the gas chromatograph could play in the microbiology laboratory in the future. The following points were made: 1) all classes of biological compounds (proteins, carbohydrates, and lipids), except for high molecular weight substances, are amenable to analysis by GLC; 2) the instrument has great sensitivity, especially when combined with an electron capture detector (the electron capture detector being more sensitive than the flame ionization detector); 3) the use of pyrolysis of whole organisms would not work well because of the complicated nature of the chromatograms generated and an intrinsic difficulty with the standardization of methodology. The authors concluded that the gas chromatograph is a powerful and versatile analytical tool capable of processing such large amounts of significant data that its invasion of the clinical microbiology laboratory is unavoidable.

In 1970, Mitruka *et al.* (88) demonstrated that the rapid detection of bacteremia in mice was possible by GLC analysis of serum taken from the infected animals. Again, they were

able to show that metabolic products from in vitro studies paralleled those obtained from infected animals. However, the inoculum used for the injection of each animal was extremely high (10^4 - 10^7 bacteria/ml of blood and 10^4 - 10^6 bacteria per gram of tissue) which may have accounted for the heavy load of metabolic products detected in the serum. Brooks (13) investigated the use of GLC in the diagnosis of the causes of arthritis. The chromatograms obtained from both acidic and basic extracts of synovial fluid were sufficiently dissimilar to allow differentiation between synovitis caused by trauma from that caused by staphylococcal, streptococcal, and gonococcal infections. It was felt that the technique might prove useful in determining the etiology of various types of arthritis.

Spinal fluid has been analyzed for the presence of neutral sugars in hopes of detecting abnormal levels when an infection was present (6). Derivatization by silylation was used to make compounds sufficiently volatile for detection by flame ionization. However, reducing sugars, due to their ability to form anomers, gave multiple peaks with this type of derivatization procedure. It was found that normal cerebrospinal fluid gave six major component peaks. Peaks 5 and 6 were identified by mass spectroscopy to be the alpha and beta anomers of glucose. In cerebrospinal fluids from patients with cryptococcal meningitis, there was a loss of peaks 1-4 and the appearance of a peak 7. However, the

identity of peak 7 was not determined. Also, the sample size was too small to determine the significance of these findings concerning the use of gas-liquid chromatography to diagnose cryptococcal meningitis by the silylation of cerebrospinal fluid and subsequent analysis by GLC.

Recently, Brooks (14) and Davis and McPhearson (30), have reviewed various methodologies and equipment involved in analyzing infected body fluids by GLC. In each paper it was stated that gas-liquid chromatography was a rapid and sensitive technique that should be very useful in the diagnosis of both bacterial and fungal diseases.

Anaerobic infections have been diagnosed by GLC examination of pus (100). The GLC analysis of pus allowed presumptive differentiation of anaerobic versus aerobic infections. Specific identification of the anaerobes causing the infection was not possible.

Gas-liquid chromatography has recently been used to diagnose endocarditis caused by beta hemolytic streptococcus group B (81). The gas-liquid chromatogram obtained from the patient's serum was different from those obtained from normal sera and from patients with proven invasive candidiasis. The chromatograms closely mimicked chromatograms generated from culture fluid in which the organism had been grown.

Some research groups, instead of pattern matching, have chosen to look for the presence of a particular metabolite

in body fluids. Two groups (12, 46) looked at cerebrospinal fluid for the presence of lactate. Lactate levels were found to be elevated in patients with bacterial and tubercular meningitis as opposed to the levels found in cerebrospinal fluid of patients with viral meningitis or in the control group. Lactate concentrations were also elevated in the cerebrospinal fluid of patients with non-infectious disorders of the central nervous system. However, if the investigator used the data obtained concerning the lactate levels in conjunction with conventional laboratory data and clinical observation, an increase in the reliability of the method for the rapid diagnosis of bacterial meningitis was found. Significantly, central nervous system infections due to viral agents were not found to elevate the lactate levels.

Gas-liquid chromatographic analysis of body fluids from patients with fungal disease has been directed toward the diseases caused by both Candida albicans and Cryptococcus neoformans. Schlossberg et al. (111) demonstrated the possibility of diagnosing cryptococcal meningitis by a GLC technique. Chromatograms obtained from cerebrospinal fluid of eight patients had peaks that were similar in size, shape, and retention times. The abnormal chromatograms also matched those obtained from nutrient broth cultures in which Cryptococcus neoformans was grown. Craven et al. (28) confirmed these results when they looked at the chromatograms obtained from cerebrospinal fluid specimens from patients with

tubercular, cryptococcal, and viral meningitis. The chromatograms were sufficiently dissimilar to allow differentiation of the cause of the patient's meningitis as being either tubercular, cryptococcal, or viral in origin. Brooks et al. (15) extended Craven's work and found that 3-(2'-ketohexyl) indoline was specifically associated with tubercular meningitis and was not found in either cryptococcal or aseptic meningitis. However, extraction and derivatization techniques used in both studies required a great deal of practice to insure reproducibility. Additionally, the sample had to be anhydrous because the electron capture detector is very sensitive to the presence of water.

The use of GLC analysis of body fluids for the diagnosis of candidiasis has been approached from two directions: derivatization of a serum with analysis of the GLC chromatograms for particular patterns and derivatization of serum followed by a search for the presence of a particular metabolite. Miller (86) analyzed chromatograms obtained from derivatized human serum. The chromatograms attained from normal sera contained peaks consisting of methyl esters of palmitic, oleic, linoleic, and steric acids. Derivatization of serum from patients gave chromatograms with the same peaks as those obtained from derivatized normal serum plus some additional peaks. Retention times of the new peaks corresponded to peaks found in chromatograms obtained from the derivatization of both broth cultures and serum cultures

of Candida albicans. The chromatograms of sera from patients with candidiasis were readily distinguishable from chromatograms obtained from the derivatized normal sera as well as sera from patients with mucosal candidiasis. Monson and Wilkinson (90, 91) found that the mannose levels in patients with candidiasis were significantly higher than those levels in normal individuals. This finding allowed for the diagnosis of candidiasis by the gas-liquid chromatographic analysis of patient serum for the presence of high concentrations of mannose.

Kiehn (72) looked at sera from patients with candidiasis and was able to show the presence of arabinitol in the sera. Diagnosis of candidiasis by GLC analysis of serum for the presence of arabinitol was demonstrated.

The initial work using arabinitol as a marker metabolite for detection of candidiasis was followed by the testing of a procedure in which specific ion monitoring was used to quantitate the arabinitol levels in patients' sera (105). The extraction of the sera and the derivatization of the extract was the same as the procedure employed by Kiehn et al. (72). By using mass spectrophotometric monitoring of selected ions of arabinitol and an internal standard, the GLC analysis of patients' sera was given the added advantages of specificity and quantification of small incremental changes in concentration. The results obtained with this methodology closely approximated the results obtained by

Kiehn et al. (72). It appeared that analysis of patient sera for the presence of arabinitol was useful in the rapid diagnosis of candidiasis. However, Eng et al. (40) presented data from both animal and patient sera which showed that arabinitol was not always an accurate marker for candidiasis due to its variable concentrations in the serum of both animals and humans with candidiasis. These authors concluded that elevated serum levels of arabinitol in a patient who had no evidence of either renal failure or bacterial infection did indicate candidiasis, but in patients without elevated serum arabinitol levels or with signs of renal failure, the lack of elevated serum arabinitol levels did not mean that the patient did not have candidiasis. Thus, more work needs to be done to make this a reliable and rapid diagnostic method for candidiasis.

Coccidioides immitis cell walls have been shown to contain an unusual reducing sugar, 3-0-methyl mannose (102, 110). Following the lead of Kiehn (72), this unusual reducing sugar may serve as a marker in the GLC diagnosis of coccidioidomycosis.

CHAPTER III

MATERIALS AND METHODS

Fungal Strains

The following strains were obtained from the mycology stock culture collection at the Wadley Institutes of Molecular Medicine, Dallas, Texas: Coccidioides immitis Merritt, Coccidioides immitis McCoy, Coccidioides immitis X-243, Coccidioides immitis X-175, and Coccidioides immitis X-247. Coccidioides immitis 46 and Blastomyces dermatitidis SCB-2 were both obtained from Dr. Rebecca A. Cox of the San Antonio State Chest Hospital.

Chemicals

Chemicals used are described in the following sections dealing with the methodology used in this study. All chemicals were of reagent grade quality or better and were used without further purification unless otherwise indicated. Chemicals were obtained from diverse sources.

Fungal Antigens

Antigens obtained from Blastomyces dermatitidis included blastomycin KCB-26 (a culture filtrate) obtained from Coy Smith of the University of Kentucky, Lexington, Kentucky, and an alkali-soluble, water-soluble extract of the yeast phase

cells of Blastomyces dermatitidis SCB-2 (b-ASWS). Antigens from Coccidioides immitis included coccidioidin, (a toluene induced lysate of mycelial phase cells of Coccidioides immitis strains Merritt, McCoy, X-243, X-175, and X-247) and an alkali-soluble, water-soluble cell wall extract of the mycelial-arthroconidial phase of Coccidioides immitis strain 46 (c-ASWS). Antigens obtained from Histoplasma capsulatum included histoplasmin H-42 (a culture filtrate) obtained from the Center for Disease Control, Atlanta, Georgia, and histoplasmin HKC-43 obtained from Coy Smith, University of Kentucky, Lexington, Kentucky.

Antigen Production

Preparation of b-ASWS

Yeast phase cells, harvested from three-day-old cultures on brain heart infusion agar slants, were inoculated into flasks containing brain heart infusion broth. The broth cultures were incubated at 37 ^{o} C in a gyratory shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) with constant shaking at 120 rpm. After 72 hours of growth, merthiolate was added to a final concentration of 1:10,000, and the cultures were placed at 4 ^{o} C for 24 hours. The merthiolate-killed cells were harvested by centrifugation (5,000 x g, 10 minutes), and then, washed with distilled water to remove the bulk of the medium constituents. The washed yeast phase cells were resuspended with an equal

volume of glass beads (0.45-0.55 mm in diameter), and then, shaken in a Braun model MSK homogenizer for 90 seconds at 2,000 rpm. A carbon dioxide cooling device was used to minimize heat during breakage. The homogenized cell suspension was centrifuged at 27,000 x g for 10 minutes in a Sorvall model RC-5 refrigerated centrifuge. The supernatant was decanted carefully and discarded. The cell wall pellet was resuspended in distilled water, centrifuged, and the supernatant was discarded. This process was repeated 5 times.

Cell walls were partially freed of cytoplasmic constituents by incubation in a 0.01 M potassium phosphate buffer (pH 7.2) containing trypsin (100 mg/ml). The trypsin treated walls were hydrolyzed in 1 N NaOH (1 ml/mg) for 3 hours in a water bath shaker at 25 ^{o} C. After hydrolysis, a suspension was centrifuged for 10 minutes at 27,000 x g, 4 ^{o} C, in a Sorvall model RC5 refrigerated centrifuge (Du Pont Company, Biomedical Products Division, New Town, Connecticut). The alkali-soluble supernatant was decanted, passed through a membrane filter (0.45 µm pore size, Millipore Corporation, Bedford, Massachusetts), then dialyzed against several changes of distilled water at 4 ^{o}C (final pH 7.0). The alkali-soluble, water-soluble glucan which precipitated during dialysis was removed by centrifugation. The supernatant [alkali-soluble, water-soluble (b-ASWS)] was filtered and then lyophilized and stored at -20 ^{o} C.

Preparation of c-ASWS

Growth for fractionation was obtained by inoculating 10 arthroconidia (estimated by hemocytometer count) suspended in sterile physiological saline into a 2-L Erlenmeyer flask containing 1-L of a 1% yeast extract-2% glucose broth. After 96 hours of incubation in a gyratory shaker (New Brunswick Scientific, New Brunswick, New Jersey) at 30 ^{o}C, 13.5 ml of a 40% formaldehyde solution was added to each culture. The flask was then placed at 4 ^{o}C for 24 hours. The formalin-killed mycelium and arthroconidia were harvested by filtration through a number forty Whatman filter paper disc (Whatman Laboratory Products, Inc., Paper Division, Clifton, New Jersey), and washed 3 successive times by resuspending the mycelial-arthroconidia mat in distilled water and centrifuging at 10,000 x g for 5 minutes. The mat was then resuspended in distilled water to approximately 10 times its packed volume, mixed with an equal volume of glass beads (0.45-0.55 mm i.d.) and shaken in a Braun model MSK (B. Braun Instruments, San Francisco, California) homogenizer for 120 seconds at 2,000 rpm. Heating was minimized during the breakage with a carbon dioxide cooling device. The homogenized suspension was centrifuged for 10 minutes at 27,000 x g, 4 ^{o}C, in the refrigerated centrifuge. The supernatant was discarded, and the cell wall pellet was resuspended in distilled water and washed 3 times by centrifugation at 27,000 x g for 10 minutes at 4 ^{o}C.

To further purify the cell walls of cytoplasmic material, the cell wall pellets were resuspended in a 0.01 M potassium phosphate buffer (pH 7.2), containing 100 mg/ml of trypsin (Sigma Chemical Company, St. Louis, Missouri) to approximately 10 times their packed volume. The suspension was incubated for 3 hours in a water bath shaker (New Brunswick Scientific Company, Inc., Edison, New Jersey) at 30 ^{o}C. The trypsin treated cell walls were washed in distilled water by centrifugation at 27,000 x g for 10 minutes at 4 ^{o}C until the filtered supernatant had an absorbance of 0.005 or less at a wave length of 280 nm with a Gilford model 540 Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The brown, non-cell wall material was removed from the upper surface of the cell wall pellet after each wash with a spatula. The trypsin treated cell walls were extracted with 1N NaOH (1 ml/mg of cell wall material) for 3 hours at 25^{o} C. After the extraction was over, the cell wall suspension was centrifuged at 27,000 x g for 10 minutes at 4^{o} C, and the alkali-soluble supernatant obtained was passed through a 0.45 µm filter (Millipore Corporation, Bedford, Massachusetts). The filtrate was dialyzed against several changes of distilled water at 4 ^{o}C until the pH of the dialysate was 7.0. The alkali-soluble, water-insoluble precipitate that formed during the dialysis was removed by centrifugation. The supernatant (c-ASWS) was again filtered through a 0.45 µm pore size membrane (Millipore Corporation,

Bedford, Massachusetts), lyophilized and stored at -20° C.

Preparation of Toluene Lysate Antigenic Mixture

The broth medium for production of coccidioidin was prepared as follows: 240 ml of a 10% aqueous solution of yeast extract (Difco Laboratories, Detroit, Michigan) was dialyzed against a 10-fold volume of distilled water at 80-90 °C for 6 hours. The residue was discarded and the dialysate was made up to the original volume. Glucose was then added to a final concentration of 2%, and the medium was dispensed in 100 ml aliquots into 500-ml Erlenmeyer flasks. The flasks were then autoclaved at 121 °C, 15 pounds of pressure for 15 minutes. Flasks of broth were then inoculated with a small piece of a fungus mat of one strain of C. immitis taken from an agar slant and incubated at 32-34 °C in a stationary manner while placed in a gyratory shaker (New Brunswick Scientific Company, Inc., Edison, New Jersey) for 48 hours. After the stationary period, the flasks were rotated for 72 hours at 50 revolutions per minute.

After the full period of growth, the fungal mats were collected on a double layer of Whatman number one filter paper (Whatman Laboratory Products, Inc., Paper Division, Clifton, New Jersey) by filtration through a Buchner funnel. The pooled mats were transferred to an Erlenmeyer flask and made into a thick slurry with distilled water. To 100 ml of slurry was added 3 ml of toluene. The flasks were tightly

stoppered, shaken, and incubated at 37 ^{o}C for 3 days. The pasty fungus mass was separated by filtration through a Buchner funnel and discarded. Aqueous merthiolate was added to the filtrate to a final concentration of 1:10,000 and the amber colored fluid was sterilized by passage through a 0.22 μm filter (Millipore Corporation, Bedford, Massachusetts). The pooled toluene lysate was used without further purification.

High Performance Liquid Chromatographic Analysis of Fungal Antigens

Apparatus

All high performance liquid chromatographic (HPLC) separations, except for the reverse-phase chromatographic work, were performed on a modular instrument consisting of a Glenco dual head pump model DHP-16, a model NRS-6 nitrogen pressure reservoir system, a Rheodyne model 7125 injection valve, an LKB ultragrad gradient maker, and an Isco model 1840 variable wave length detector. All chromatographic data were recorded and integrated by a Spectra-Physics 4100 computing integrator. The reverse-phase work was performed on a Spectra-Physics SP8100 automated liquid chromatograph with a model 8440 uv/vis variable wave length detector. The chromatographic system was interfaced with the SP4100 computing integrator which was used to control the

chromatograph, as well as to record and integrate the data obtained from the chromatographic run.

Columns

For the gel permeation chromatographic (GPC) analysis, two different columns were used: (1) a 25 cm x 4.6 mm i.d. stainless steel column was dry packed with a glycerylpropyl control pore glass with a nominal pore size of 500 Å (Electro-Nucleonics, Fairfield, New Jersey); (2) a glyceryl-propyl layer covalently bonded to Lichrosphere silica particles (10 μ) which was purchased prepacked in a 25 cm x 4.1 mm i.d. stainless steel column from Synchrom, Inc. (Linden, Indiana). The nominal pore size of this packing was 300 Å.

For ion-exchange chromatography, a 25 cm x 4.6 mm i.d. stainless steel column was dry packed with DEAE-control pore glass (Electro-Nucleonics, Fairfield, New Jersey).

The reverse-phase analysis was accomplished with a 10 cm x 4.1 mm i.d. stainless steel column purchased prepacked from Synchrom packed with a 6.5 μ silica to which a C8 phase was covalently bonded. The nominal pore size of the packing is 300 Å.

Mobile Phase

The mobile phases used in the GPC analysis were either a 0.015 M ammonium bicarbonate, pH 9.6, or 0.25 M phosphate buffer, pH 7.8. For the ion-exchange chromatographic work,

the primary mobile phase was a 0.02 M tris acetate buffer, pH 7.9, with an eluting buffer (solvent B) that was 0.02 M tris acetate containing 4 M sodium acetate, pH 7.9. The elution of proteins that had remained on the column was accomplished through the use of a linear gradient running from 0-100% solvent B. In the reverse-phase analysis, protein elution was accomplished by the application of a binary gradient in which the primary mobile phase was 0.013 M (0.1% TFA, v/v) trifluoroacetic acid (TFA) in water, and a linear gradient was generated from 0-60% of solvent B which was 2-propanol in 0.013 M TFA.

Analytical Methods

A small amount of dried c-ASWS was mixed with dry potassium bromide and pressed into a potassium bromide disc. The absorption spectrum (4,000 - 250 cm^{-1}) was determined using a Perkin Elmer model 457 grading infrared spectrophotometer.

The amount of lipid contained in c-ASWS was determined as follows: 1 gram of c-ASWS was extracted with chloroform-methanol 2:1 (v/v). The c-ASWS was combined with 20 ml of the chloroform-methanol 2:1 (v/v) and extracted with stirring for 5 minutes. The insoluble material was sedimented by centrifugation and the supernatant removed. The insoluble material was extracted twice more. After each extraction, the solution was filtered through a Buchner

funnel, and the residue was resuspended for further extraction. The 3 filtrates were combined, and then, the solvent was removed under vacuum. The lipid obtained was redissolved in a minimal amount of chloroform-methanol, placed into a pre-weighed planchette, and then, placed in a desiccator over potassium hydroxide, and dried under vacuum. After drying, the weight of the planchette was determined, and the difference between the measured weight before and after addition of the lipid material was taken as the weight of the lipid extracted.

The amount of carbohydrate present in c-ASWS was determined by a colorimetric method using phenol-sulfuric acid reagent. Two ml of a solution containing 1 mg of c-ASWS was pipetted into a test tube, and 0.05 ml of 80% phenol was added. Then 5 ml of concentrated sulfuric acid was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube, in order to obtain good mixing. The tubes were allowed to stand for 10 minutes, then they were shaken and placed for 10-20 minutes in a water bath at 25 ^{o} to 30 ^{o}C before readings were taken. The absorbance was read at 490 nm with a Perkin Elmer model 200 spectrophotometer. Blanks were prepared by substituting distilled water for the sugar solution. The amount of sugar was then determined by reference to a standard curve previously constructed using mannose as the reference sugar.

The amount of protein present in c-ASWS was determined by colorimetric methods using the methodology of Lowry (78). Briefly, 1 mg of c-ASWS was dissolved in 1 ml of 0.1 N sodium hydroxide. A 0.1 ml aliquot was added to each of 3 tubes; then, 0.7 ml of 0.71 N sodium hydroxide plus 2 ml of Lowry's solution D were added per tube, and all samples were kept at room temperature for 10 minutes. To each tube, 0.2 ml of dilute (1:1) Folin-Ciocalteu reagent was added with rapid mixing and absorbance was read at 650 nm after 30 minutes. The amount of protein was then determined by referring to a reference curve previously constructed using bovine serum albumin as a standard.

Amino Acid Analysis

To 1 mg of c-ASWS placed in a hydrolysis tube was added 0.2 ml of 6 N HCl containing 4% thioglycolic acid and 0.1% phenol. The tube was then sealed under vacuum. The tube was placed in a temp block heater at 110 ^{o}C for 24 hours. After hydrolysis, the tube was cooled, opened, and the contents taken to dryness over phosphorous pentoxide and sodium hydroxide pellets under vacuum. The residue was dissolved in 0.5-1.0 ml of 0.2 M sodium citrate (pH 3.25). Then, 50 μl of this solution was injected into a Glenco amino acid analyzer for analysis. Detection was accomplished with ninhydrin and a dual wavelength filter photometer (440/570 nm). A mixture of standard amino acids was run

first to ensure that complete operation of the instrument was operating properly as well as to provide an external standard for quantitation purposes.

β-Elimination Reaction

Aliquots (1.0 ml) of a solution containing from 1-5 mg/ml of c-ASWS in 0.1 M sodium hydroxide and 0.6 M sodium borohydride were placed in 150 x 20 mm teflon-lined screw capped culture tubes. The tubes were incubated at 45^{o} for 6, 10, 15, and 24 hours, and then, cooled to 20-25^{o} C in an ice bath. One drop of 1-octanol and a teflon-covered stirring bar were added to each tube. With vigorous stirring, the contents were neutralized by the addition of 1.0 ml of 0.4 M hydrochloric acid, immediately followed by 0.1 ml of 0.08 M aqueous palladium solution. Sodium borohydride (2.0 ml of a 0.66 M solution in a 0.1 M sodium hydroxide) and palladium chloride (2.0 ml of a 0.016 M solution in 0.8 M hydrochloric acid) were then added simultaneously, dropwise, from separate pipets. To each tube, 6.1 ml of concentrated hydrochloric acid was then added. The tubes were tightly capped, heated at 110^{o} C for 22 hours and cooled in an ice and water bath. The tubes were opened, and the contents were dried in a rotary evaporator under diminished pressure. The residue was dissolved in a 0.2 M sodium citrate buffer, pH 2.2. The solution was filtered, and the amino acids were determined by the use of an amino acid analyzer.

Con A Reactivity

Myco-immune immunodiffusion plates, which had a pattern of wells consisting of a central well surrounded by 6 peripheral wells, were used for two-dimensional immunodiffusion in agar gels. In performing the experiments, the concanavalin A (Con A) preparation (Sigma Chemical Company, St. Louis, Missouri) (3-5 mg/ml in 0.01 tris-0.5 M sodium chloride (pH 7.2) buffer containing $MnCl_2$ (1 mM) and $CaCl_2$ (1 mM)) was added to the central well, carefully filling the well to the top. Test solutions of the fungal cell wall antigen (2-2.5 mg/ml), in the same buffer as the con A, were added to the peripheral wells again, completely filling the well. The lid was replaced, and the plate was stored at room temperature, avoiding any draft or vibrational disturbances. Precipitation bands appeared within 12 to 24 hours. The bands were visualized with a light source of incident light.

N-Terminal Analysis Using Dansyl Chloride

Labeling of the Protein

Approximately 50-250 µg of protein were placed in a small ampule, and 50 µl of 1% SDS solution were added. The protein was then dissolved by heating the mixture in a boiling water bath for 2-5 minutes. After heating, the protein solution was dried with a stream of dry nitrogen. Once dried, the protein was resuspended in 1 ml of 40 mM lithium carbonate buffer (pH 9.5, with HCl). The protein

was then labeled by the addition of 1 ml of dansyl chloride solution (1.5 mg/ml, 5.56 mM dansyl chloride in acetonitrile), gently shaken for 2 minutes, and then, allowed to stand at room temperature for 35 minutes. The reaction was terminated by adding 100 µl of 0.2% ethylamine solution.

Hydrolysis

After termination of the labeling reaction, the reaction solution was dried under a stream of dry nitrogen. 100 µl of 6.1 N HCl were added to each ampule. The ampules were sealed under vacuum and hydrolyzed for 22 hours at 110° C. At the end of the hydrolysis period, the tubes were opened, and the HCl was removed *in vacuo* over sodium hydroxide pellets.

Identification of Dansyl Amino Acids

The dansyl amino acids were dissolved in 50 µl of 50% pyridine. The dissolved sample hydrolysate (1-5 µl) was spotted on the corner of one side of a 15 x 15 cm polyamide sheet. Approximately 1 µl of a dansyl amino acid standard solution was spotted on the reverse side of the polyamide sheet. The identification of the dansyl amino acids was performed by two-dimensional thin layer chromatography. Ascending chromatography was performed with solvent 1 [water-pyridine-formic acid (93:3.5:3.5, v/v)]. After development of 12 cm, the sheet was air dried and developed in the second dimension in solvent 2 (benzene-acetic acid, 4.5:1, v/v). Identification of the unknown dansyl amino acids was carried

out after uv elimination (long wavelength) by referring to the coordinates of the dansyl amino acid standards on the opposite side of the TLC plate.

Characterization of the Polysaccharide Portion of c-ASWS by Gas Liquid Chromatography

Hyrolysis and Preparation of Samples

Samples, 1-5 mg, were hydrolyzed in 1 ml of 2.5 N trifluoroacetic acid in a temp block heater at 100° C in an evacuated sealed tube. The hydrolysis times chosen were 4, 7, and 10 hours. After hydrolysis, the tube was cut and 100 µl of a 1 mg/ml aqueous solution of D-lyxose were added to serve as the internal standard. The mixture was washed out with an equal volume of water into a 5-ml pear shaped flask. The solution was evaporated to dryness under reduced pressure at 40°, and the residue was redissolved in a small amount of water. The solution was then passed through a column containing Dowex 50 x 8 - 200 (H^+). The microcolumn was prepared by vertically positioning a 16-cm Pasteur pipet and wedging a small amount of glass wool in the tip. The resin solution was added to the microcolumn so that the bed volume was approximately 30% of the column volume. Once the excess water had drained, the resin was washed with 5 ml of water. The hydrolysate was added to the resin, followed by (a) approximately 2 ml of water, and (b) 4 ml of methanol. The effluent was collected into a 5-ml pear shaped flask, and

then, evaporated under reduced pressure at 40 °C. The column was then eluted with 10 ml of 2 N HCl to remove bound hexosamines. This effluent was collected and evaporated under reduced pressure at 40 °C. To the residues containing the hexosamines was added 1 ml of 2 M TFA at 0°. The hexosamines were then deaminated by adding an equal, millimolar amount (150 μg) of sodium nitrite. After one hour, residual nitrous acid was removed by placing the sample in a boiling water bath for 5 minutes, cooling and evaporating to dryness under reduced pressure. Samples were next converted to per-o-acetylated aldonitrile (PAAN) derivatives. For the neutral sugar residues, 0.2 ml of pyridine and hydroxylamine hydrochloride (60% of the sugar by weight) were added, and the mixture was then heated, with stirring, at 70 °C for 20 minutes in a silicone oil bath. Acetic anhydride (0.1 ml) was then added, and the heating/stirring are continued for a further 20 minutes. The reaction mixture was then cooled for a few minutes. The hexosamine residue was derivatized in the same way except that 1 ml of pyridine and 0.5 ml acetic anhydride were used. After cooling following the derivatization, the reaction mixtures were extracted in the following sequence: Four 1.3 x 10 cm test tubes were arranged--tube 1 containing chloroform (0.8 ml) and water (2.0 ml), tube 2 containing only water (2 ml), tube 3 containing a few pellets of 4 Å molecular sieve, and tube 4 empty. The reaction mixture was then transferred to tube 1

(with a 0.2 ml chloroform wash of the reaction tube), and the resulting chloroform/water mixture was mixed on a vortex mixer for 20 seconds. Once the 2 solvent phases were clearly reestablished, the chloroform phase was transferred to tube 2 and the mixing repeated. The resulting chloroform phase was then transferred to tube 3, left over the 4 Å molecular sieves for 1 hour; then, transferred to tube 4, which was stoppered with a teflon-lined screw cap. Before injection, the chloroform was evaporated by nitrogen and heat. The residue was redissolved in a small amount (20-50 µl) of chloroform, and 3 µl were injected into the GLC for analysis. A column (2 mm i.d. x 1.23 m) of 2% OV-17 on Chromsorb WHP (80-100 mesh) with a nitrogen flow rate of 22 ml/minute was employed. The temperature program was from an initial temperature of 130 to 280 ^{o}C at 5 ^{o}/min. The injector and detector were held at 280 ^{o}C. The gas chromatograph was the same one used for the analysis of human serum samples.

Molecular Weight Determination by Gel Chromatography In Guanidine Chloride

Preparation of Solvent

The eluting solvent, 6 M guanidine hydrochloride (GuHCl) was prepared by dissolving pure GuHCl (Aldrich Chemical Company, St. Louis, Missouri) into water. The solution was filtered through a Durapore membrane filter (Millipore Corporation, Bedford, Massachusetts) and degassed. The pH was adjusted to around 6.

Column Preparation

Approximately 200 grams of a Sepharose CL-4B agarose slurry (Pharmacia Fine Chemicals, Piscataway, New Jersey) was weighed out and an amount of water equal to 1/4 of the weight of the gel was added. The suspension was adjusted to a guanidine hydrochloride concentration of 6 M by the addition of solid GuHCl. This was approximated by the addition of an amount of GuHCl which equalled about 5% more by weight than the weight of the suspension. The mixture was gently swirled until the GuHCl was dissolved, and then, was equilibrated overnight. The solvent was removed by gentle suction filtration of the slurry on a Buchner funnel fitted with Whatman number two filter paper. Solvent removal was continued beyond the point at which no solvent remains above the agarose. The filter cake was then removed and resuspended in about 4 volumes of a 6 M GuHCl solution by gently swirling, and the solution was degassed under vacuum.

Packing of the column with agarose was accomplished in the following manner: An LKB 2137 chromatography column of dimensions 16 mm i.d. x 950 mm in length (LKB, Gaithersburg, Maryland), was half filled with filtered, degassed GuHCl. Any air bubbles sticking to the sides or bottoms of the column were removed with the aid of a glass rod. The column was then filled with the slurry of agarose in 6 M GuHCl. Any existing air bubbles were again removed. The solvent flow was not started until a 2-3 cm layer of agarose had

accumulated in the bottom of the column. The column effluent was collected in a beaker at the same height as the column bottom. The column was packed under solvent flow to within 5 cm of the top of the column by continuous addition of gel slurry. Once the column was packed, the reservoir was attached, the column was adjusted to the proper height, and allowed to flow for about 2 days prior to the first sample addition. The column flow rate was 3 ml per hour at a head pressure of 110 cm.

Preparation of Samples

Ten ml of each protein were dissolved in 0.5 ml 6 M GuHCl, 0.1 M 2-mercaptoethanol, and the pH was maintained at 8.6 for 4 hours. After this time, 0.5 ml of the column's solvent, which contained 0.6% blue dextran in 0.1% DNP-alanine, was added to the protein solution. The following proteins were used as chromatographic standards: cytochrome C, myoglobin, chymotrypsinogen A, albumin (egg), bovine serum albumin, aldolase (rabbit muscle), and catalase (bovine).

Sample Application and Detection

For application of the sample to the column, flow was stopped at the reservoir, and a column three-way valve was opened. The sample (0.5 ml volume) was added to the column by gravity. After the sample had layered on top of the column material, the three-way valve was repositioned to

allow solvent flow from the reservoir.

In order that accurate determinations of distribution coefficients were obtained, the volume of effluent preceding the position of maximal concentration of each species was accurately determined. This was achieved by using effluent weight rather than volume as a measured parameter. This was achieved by weighing 13 x 100 mm test tubes before and after collection of the effluent. Since solvent density is uniform, elution positions based on elution weight were precisely determined in this manner. A fraction size of 0.7-1 gram was found to be optimal.

The determination of elution position was accomplished by means of spectrophotometric monitoring for protein at 280, blue dextran at 630 nm, DNP-alanine at 360 nm, and carbohydrates at 490 nm after exposure to the phenol sulfuric acid reagent for carbohydrates.

Calculations

For each of the standards as well as the unknown c-ASWS, the following calculations were done. For each protein, a value F(V) was calculated. This was calculated using the following formula: $F(V) \frac{(Ve/Vt)^{1/3} - (Vo/Vt)^{1/3}}{1-(Vo/Vt)^{1/3}}$

where Vo is equal to the elution weight of blue dextran, Vt is equal to the elution weight of the DNA-alanine, and Ve is equal to the elution weight of the protein polypeptide chain. The cube root of the molecular weight or $M^{1/3}$ was

calculated for each of the standards. Then, a linear regression analysis was run on the two values F(V) and $M^{1/3}$. This resulted in the following equation for the line obtained from the standards: Y is equal to 0.7219464 - .0074835 $M^{1/3}$. The slope of the line was a -.0074835, the intercept was 0.7219464, and the correlation coefficient was 0.9954633. In order to determine the molecular weight of the subunits of c-ASWS, a F(V) value was again calculated for each and then they were substituted into the following formula: $M^{1/3} \frac{0.7219464 - F(V)}{.0074835}$. See Appendix for further explanation of calculations.

Gas-Liquid Chromatography of Neutral Sugars in Human Serum Samples

Samples

The following human sera were subjected to neutral sugar analysis by gas-liquid chromatography (GLC): 37 sera from patients with coccidioidomycosis; 6 sera from patients with aspergillosis; 7 sera from patients with candidiasis; 7 sera from patients with histoplasmosis; and 5 sera from patients with blastomycosis. The coccidioidomycosis sera were obtained from the Arizona State Department of Health. The aspergillosis, blastomycosis, candidiasis, and histoplasmosis sera were obtained from the Center for Disease Control, Atlanta, Georgia. Twenty-five normal serum samples

were obtained from the Clinical Chemistry Department at the Granville C. Morton Hospital, Dallas, Texas.

Apparatus

A Shimadzu (Shimadzu Scientific Instruments, Columbia, Maryland) GC-4B equipped with hydrogen flame ionization detectors was used. All chromatographs were recorded and integrated by a Spectra-Physics 4100 computing Integrator. Samples were analyzed on a 2% OV17 on Chromsorb WHP (80-100 mesh, Supelco, Bellefonte, Pennyslvania) that was temperature programmed from 130-280 ^{o}C at 5 ^{o}/min. Detector and injector temperatures were maintained at 280 ^{o}C. A carrier flow rate (nitrogen) of 30 ml/min. was used.

Chemicals

Reducing sugars were used as obtained: D-lyxose, D-ribose, D-mannose, D-glucose, D-galactose (Sigma Chemical Company, St. Louis, Missouri). Reagents were used as obtained from Fisher Scientific Company (pyridine, acetic anhydride, and chloroform, all ACS grade). Hydroxylamine hydrochloride was used as obtained from Sigma Chemical Company.

Sample Preparation

The sera were removed from the freezer, thawed in running warm water, then allowed to sit and equilibrate to ambient temperature. The sera were then mixed with the

vortex mixer for 15 seconds. Five hundred µl of the sera were then pipetted into a 16 x 100 mm Pyrex screw top tube. Five ml of 95% methanol were added to each tube, and the contents mixed for 30 seconds with the vortex mixer. Samples were then allowed to stand at ambient temperature for 15 minutes before centrifuging at 1500 g for 15 minutes. The supernatant was carefully removed, and the precipitate discarded. Supernatants were then evaporated to dryness with dry nitrogen and a tent block heater set at 40° C.

Sample Derivatization

To each sample residue was added 200 µl of pyridine containing 100 µg of hydroxylamine hydrochloride, 50 µg of ribose, and 50 µg of lyxose. The tubes were then subjected to heating and stirring at 70° for 20 minutes. Then, 100 µl of acetic anhydride were added, and the heating and stirring continued for an additional 20 minutes. The reaction mixture was then partitioned between chloroform (1 ml) and water (2 ml), and the chloroform layer was removed, washed with water, dried (Linde type 4 Å molecular sieve) for 10 minutes and then the chloroform was removed by evaporation with a stream of dry nitrogen. For analysis, the residue was redissolved in 20 µl of chloroform and 3 µl aliquots were injected into the GLC for analysis.

Synthesis of 3-O-Methyl Mannose

Preparation of Methyl 4,6-O-Benzylidene-α-D-Mannopyranoside

Finely powdered methyl- -D-mannopyranoside (40 g) was dissolved as rapidly as possible in 90-100% formic acid (200 ml). Immediately after this step, redistilled benzaldehyde (200 ml) was added to the solution. After 5 minutes of stirring, the mixture was poured with stirring into a mixture of light petroleum (boiling point 60-180°; 1600 ml) and water (1600 ml) containing potassium carbonate (550 g; anhydrous). The inorganic material which was separated from the aqueous phase was discarded. The upper layer was then filtered, and the residue was washed with light petroleum. The desired material was then crystallized from a mixture of chloroform benzene and resulted in a compound which had a melting point of 140-143°.

Preparation of 3-O-Methyl-D-Mannose

A suspension of methyl 4,6-O-benzylidene-α-D-mannopyranoside (0.2 g, 0.7 mM) and dibutyltin oxide (0.18 g, 0.7 mM) in methanol (25 ml) was heated under reflux for approximately 1 hour (clear solution), and then, the solvent was removed under diminished pressure. The resulting methyl 4,6-O-benzylidine-2,3-O-dibutylstannylene-α-D-mannopyranoside was dried under vacuum using an oil pump, taken up in N,N-dimethylformamide (5 ml), and treated with 10 domethane (0.3

ml, 4.8 mM). After the mixture had been heated for approximately 4 hours at 45 °, TLC (silica gel, 19:1 chloroform-acetone) showed complete disappearance of the starting material. Following evaporation of the solvent under diminished pressure, the material was chromatographed on a column of silica gel (19:1 chloroform-acetone). The compound that was collected from the silica gel column was methyl 4,6-0-benzylidine-3-0-methyl-α-D-mannopyranoside. 3-0-methyl-D-mannose was obtained from the preceding compound by resuspending the compound in 80% acetic acid and molar hydrochloric acid, v/v, 3:1, and heating the reaction mixture at 100 ° C for about 4 hours. Thin layer chromatography (TLC) in a developing solvent of 25% methanol and chloroform, showed one lower mobility spot with an RF = 0.3-0.4. This was 3-0-methyl-D-mannose.

CHAPTER IV

RESULTS

High Performance Liquid Chromatographic Analysis of Fungal Antigen Preparations

In Figures 1-6 are presented typical chromatograms obtained when six different fungal antigen preparations were subjected to size exclusion chromatography on a 300 Å SEC column (Synchrom, Linden, Indiana). As seen in Figure 1, the size exclusion chromatogram obtained with histoplasmin H-42 revealed two major peaks and two minor peaks on either side of the two major peaks. The major peak with a retention time of 7.07 minutes is very sharp, and this may indicate homogeneity. However, the other three peaks were much broader, and the chromatogram indicated that they overlap each other. The chromatogram further showed that this column did not give sufficient resolution to allow the separation of the various components contained in this fungal antigen mix. A chromatogram obtained from the separation of histoplasmin HKC-43 is shown in Figure 2. Unlike the chromatogram shown in Figure 1, this chromatogram contains more demonstrable peaks but even less separation than was demonstrated in Figure 1. The major peak in this chromatogram occurred at a retention time of 7.32 minutes. Again, as shown in Figure 1, resolution on this column was not good enough to allow separation of the

Fig. 1--Size exclusion chromatography of histoplasmin (H-42) using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

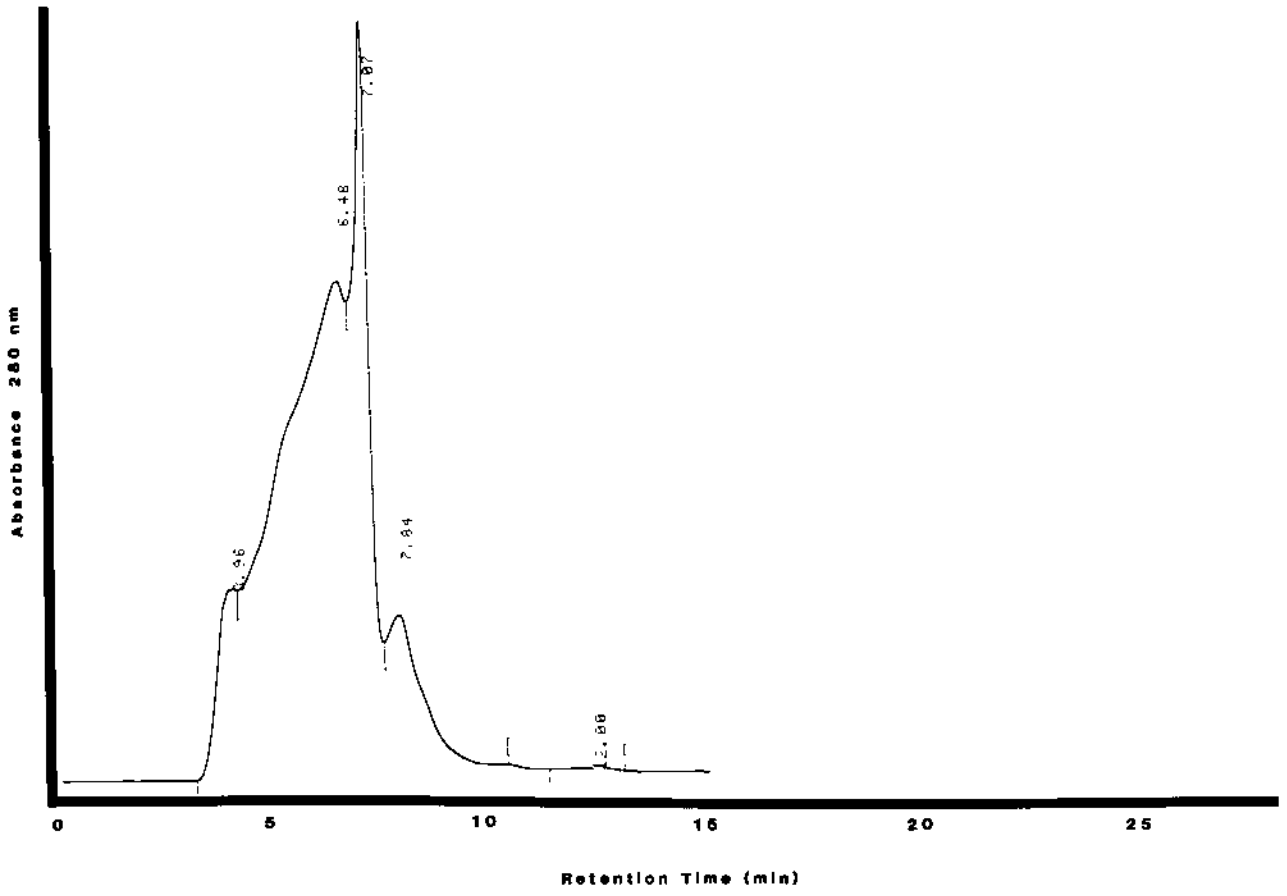
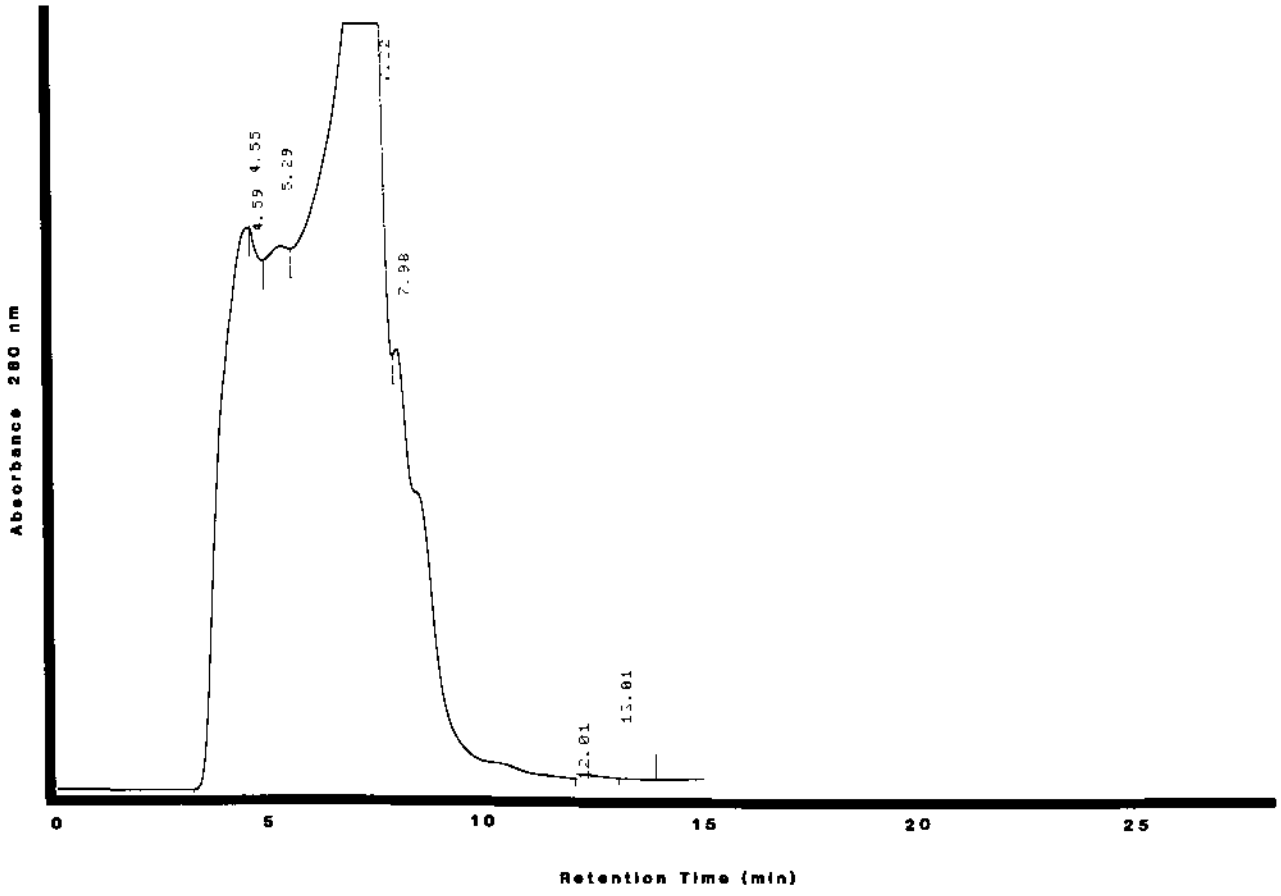

Absorbance 280 nm
5.48
7.87
7.84
12.86
0
5
10
15
20
25
Retention Time (min)

Fig. 2--Size exclusion chromatography of histoplasmin (HKC-43) using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.


Absorbance 280 nm
4.59
4.55
5.29
7.98
12.01
15.01
0
5
10
15
20
25
Retention Time (min)

five demonstrated component peaks. In Figure 3, the chromatogram of blastomycin KCB-26 shows an extremely large peak at 5.91 minutes bordered on either side by two smaller peaks. From this chromatogram, it appears that size exclusion chromatography could be used to collect the main component peak shown for blastomycin KCB-26. After collection, this peak could then be tested for its reactivity as a skin test reagent. Figure 4 clearly demonstrates that the technique of size exclusion chromatography could be used to separate at least two major and two minor peaks from the b-ASWS fungal antigen preparation. The chromatogram shows that the largest component has a retention time of 3.55 minutes with the other major component having a retention time of 5.76 minutes.

The peak shape of the component eluting with a retention time of 5.76 minutes probably indicates homogeneity for this particular component. For b-ASWS, size exclusion chromatography would be a good method to separate components for further structural and immunological testing. The size exclusion chromatography pattern of coccidioidin, which is shown in Figure 5, is fairly unremarkable in that it shows only two component peaks. This is an unexpected result, since coccidioidin is a filtrate from C. immitis strains grown in liquid culture and, as such, would be expected to contain a great number of different proteins, polysaccharides, and glycoproteins. Thus, size exclusion chromatography with this column would not be useful for

Fig. 3--Size exclusion chromatography of blastomycin (KCB-26) using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

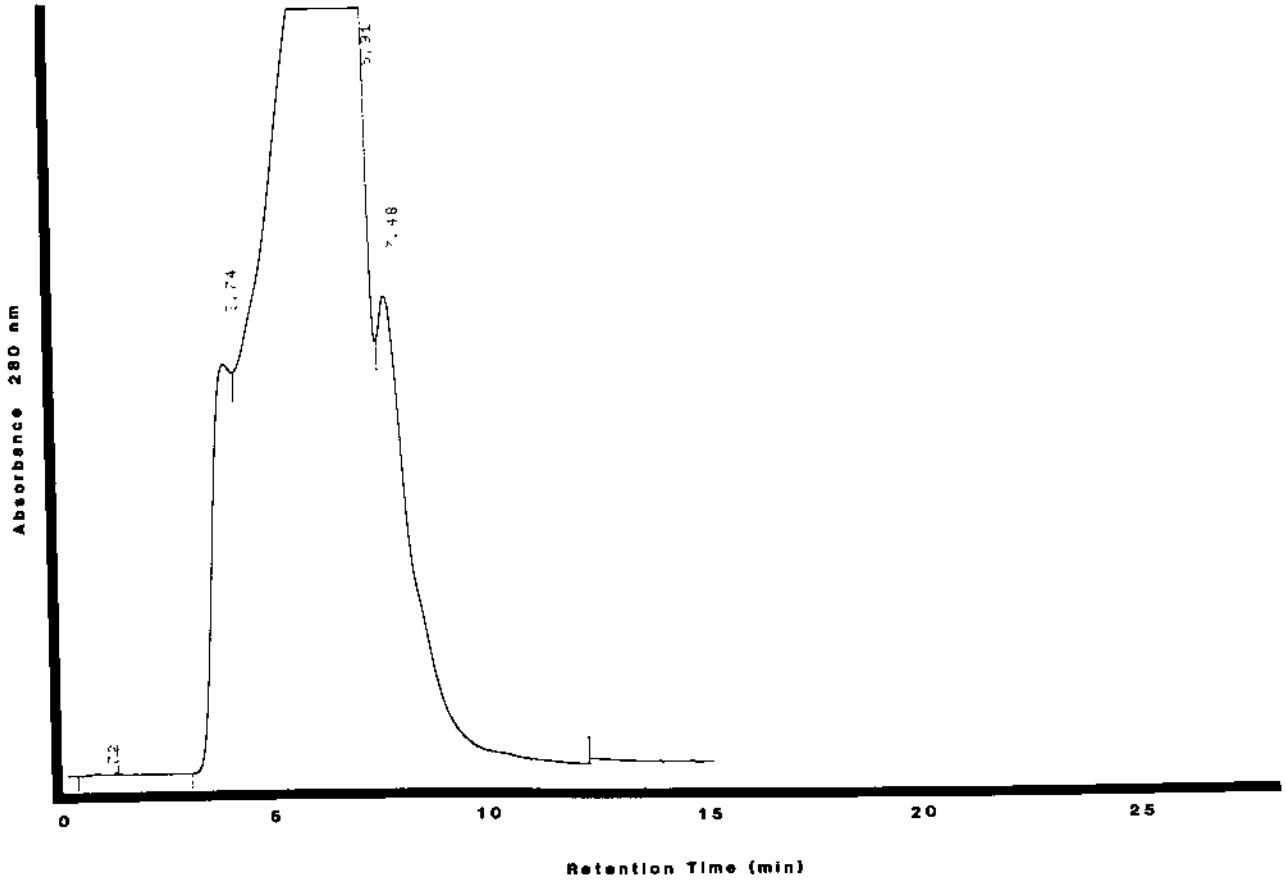

Absorbance 280 nm
3.74
5.15
7.48
0
5
10
15
20
25
Retention Time (min)

Fig. 4--Size exclusion chromatography of an alkali-soluble, water-soluble antigen of Blastomyces dermatitidis (b-ASWS) using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

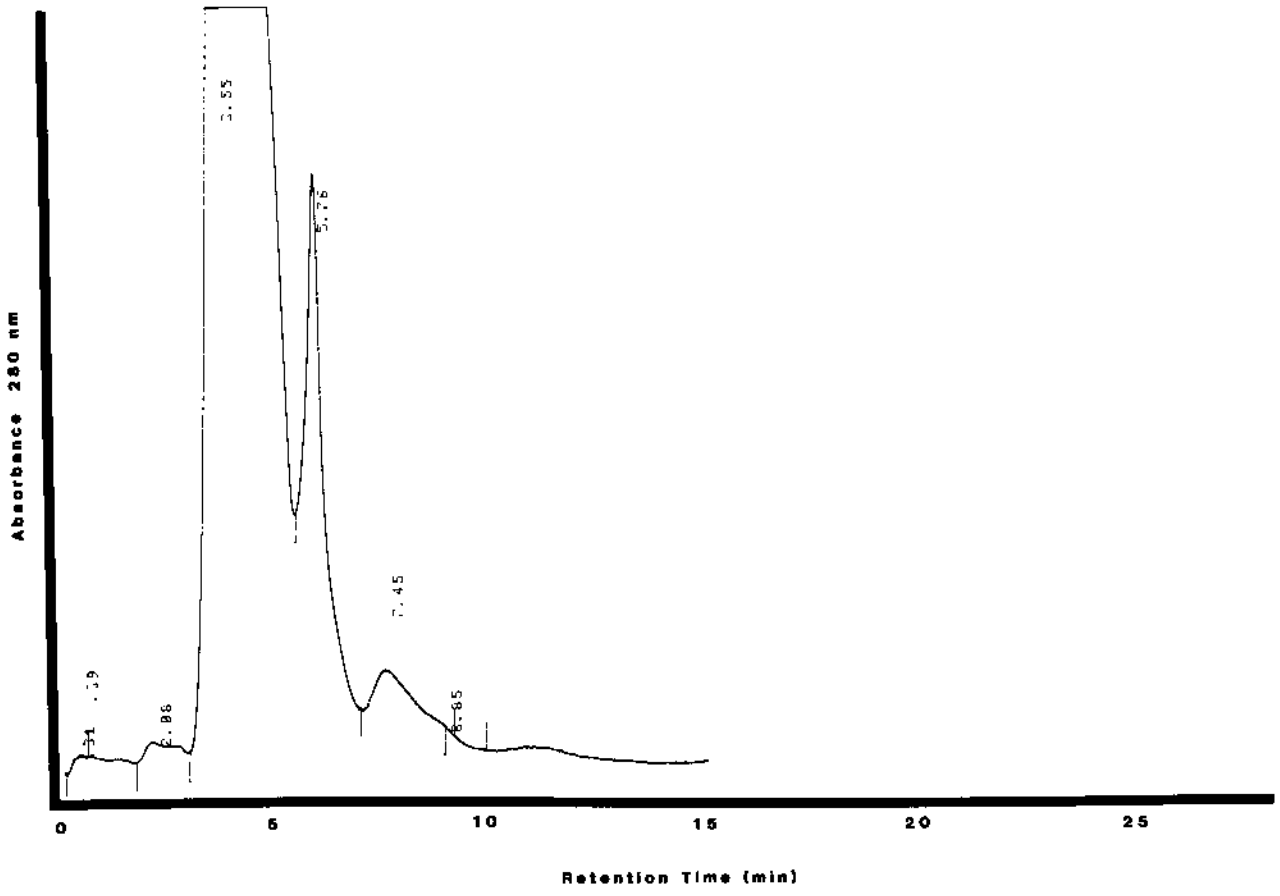
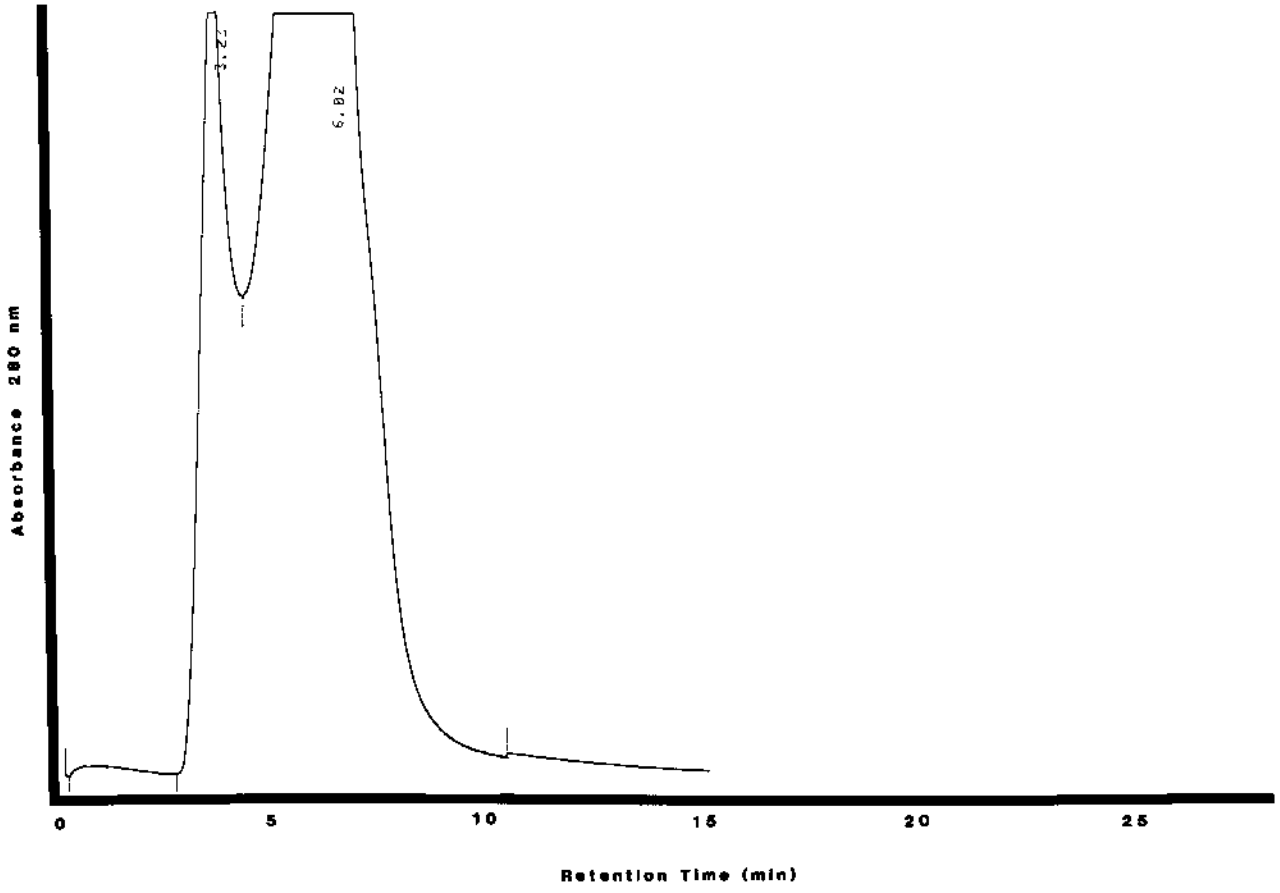

Absorbance 280 nm
3.55
5.78
7.45
6.85
6.86
0
5
10
15
20
25
Retention Time (min)

Fig. 5--Size exclusion chromatography of coccidioidin using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.


3.22
28.9
Absorbance 280 nm
0
5
10
15
20
25
Retention Time (min)

separating the components of coccidioidin. Figure 6 details the chromatographic pattern obtained when c-ASWS was subjected to chromatography on the size exclusion column. Not unlike the pattern shown for b-ASWS, it details three main peaks, one of which, the peak at 5.71 minutes, could be adequately separated from the other two overlapping peaks at retention times of 3.68 and 4.24 minutes, respectively. Further, the peak with a retention time of 5.71 minutes has the same peak shape and equivalent area as the peak at 5.76 minutes in the chromatogram (Figure 4) of b-ASWS. Additional analysis needs to be done, but it can be stated now that two peaks are at least the same molecular weight and may, in fact, be structurally similar if not the same.

To facilitate comparison of peak retention times among the six fungal antigen preparations, a schematic representation consisting of vertical bars is presented in Figure 7. The elution times of the different components of each fungal antigen preparation were remarkably constant from run to run using the same column and isocratic conditions and did not differ by more than 0.15 minutes from the expected value. None of the component peaks were shared by all six fungal antigen preparations. Both KCB-26 and b-ASWS shared a peak with a retention time of 7.45 minutes. As pointed out in the preceding paragraph, c-ASWS and b-ASWS may have a component in common which elutes in the range of 5.71-5.76 minutes. Overall, the six fungal antigen preparations tested with this

Fig. 6--Size exclusion chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

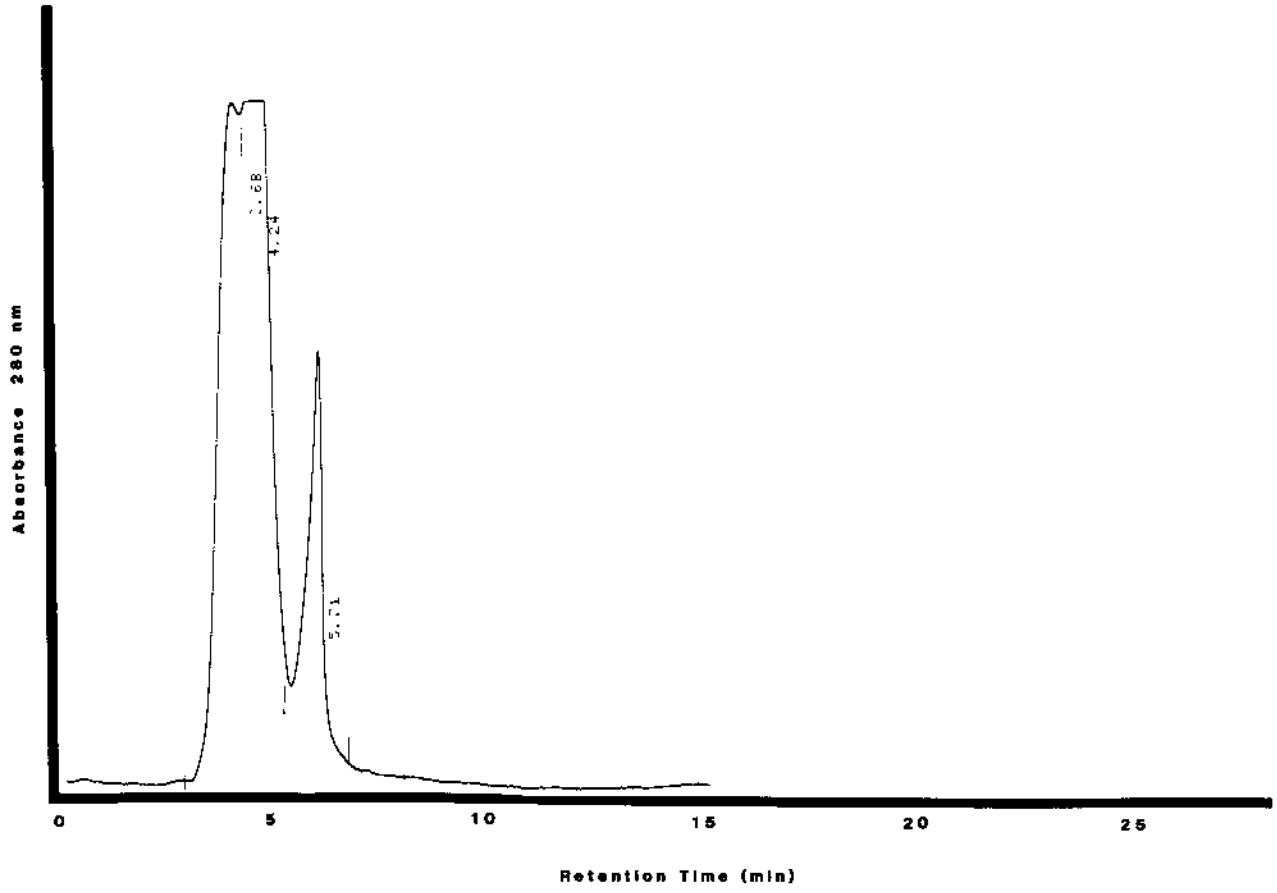
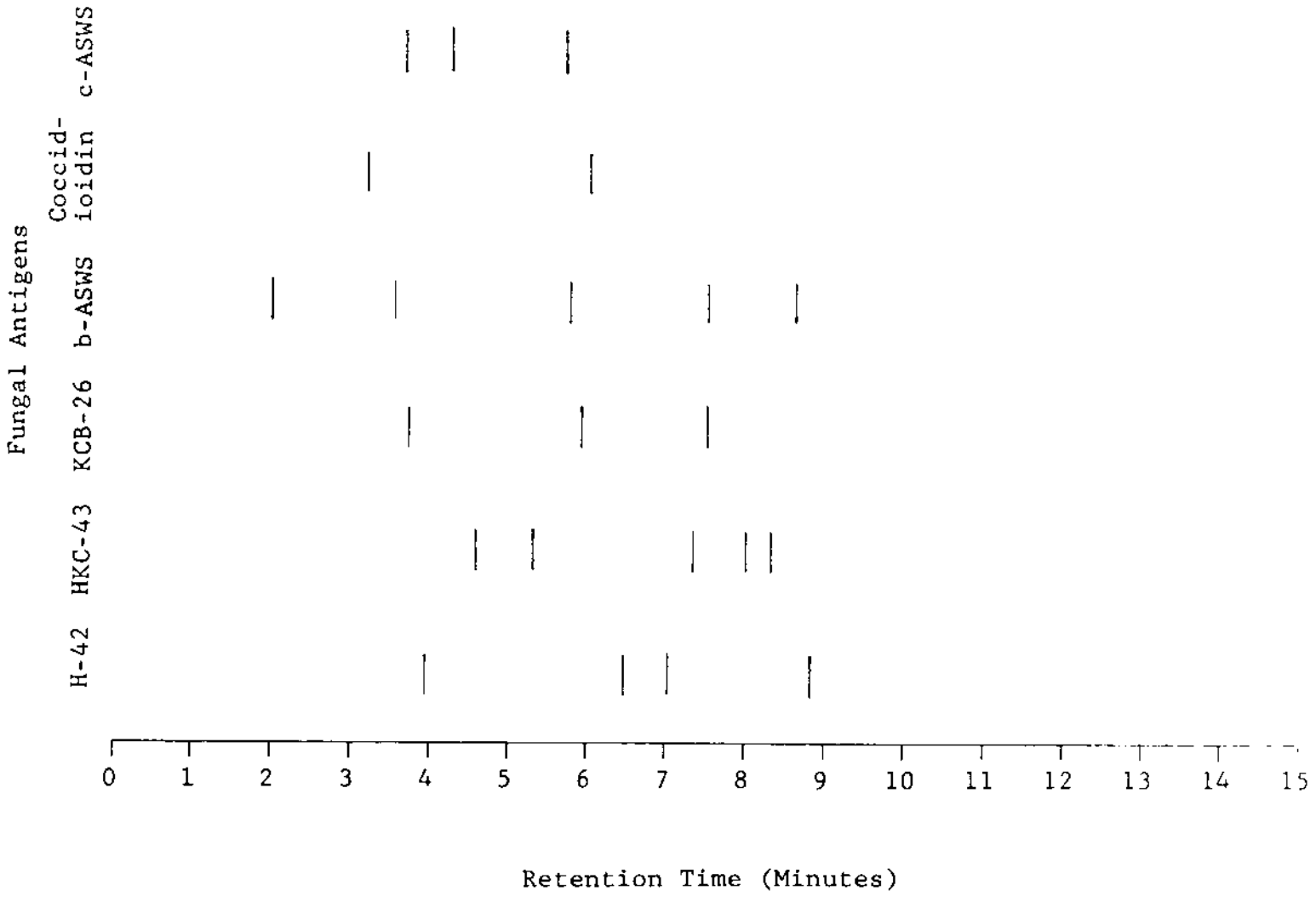

Absorbance 280 nm
0
5
10
15
20
25
Retention Time (min)

Fig. 7--Schematic representation of the protein map chromatograms of fungal antigen preparations obtained by size exclusion chromatography. The vertical bars represent the average retention times of component peaks of multiple runs (±0.15 min) for each fungal antigen preparation.


Fungal Antigens
H-42
HKC-43
KCB-26
b-ASWS
Coccid-
ioidin
c-ASWS
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Retention Time (Minutes)

SEC column appear to be composed of components which are similar in molecular weight, but which may not be structurally or immunologically similar. Further study is needed to answer these questions.

The typical patterns obtained from ion-exchange chromatography of the six fungal antigen preparations are presented in Figures 8 through 13. In Figure 8 the ion-exchange chromatographic pattern obtained from the chromatography of histoplasmin H-42 is shown. The pattern is significant in that if one refers back to Figure 1, the size exclusion chromatographic pattern, the antigenic preparation is now shown to contain at least five instead of the previously demonstrated four component peaks. The chromatogram shows that baseline separation has been achieved with two main components which were not bound to the column. Elution with the linear gradient demonstrated three overlapping components. The ion-exchange chromatographic pattern of histoplasmin HKC-43, as shown in Figure 9, demonstrates the same number of peaks as were seen in the size exclusion chromatographic pattern. Again, baseline separation was achieved for the first two components which were not bound to the column. Those peaks that were bound to the column show, once again, very poor separation. Figure 10 shows the ion-exchange chromatographic pattern obtained with blastomycin KCB-26. This pattern is quite different from the one obtained by size exclusion chromatography (Figure 3) in that it shows, as with

Fig. 8--Ion-exchange chromatography of histoplasmin (H-42) using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 Å with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

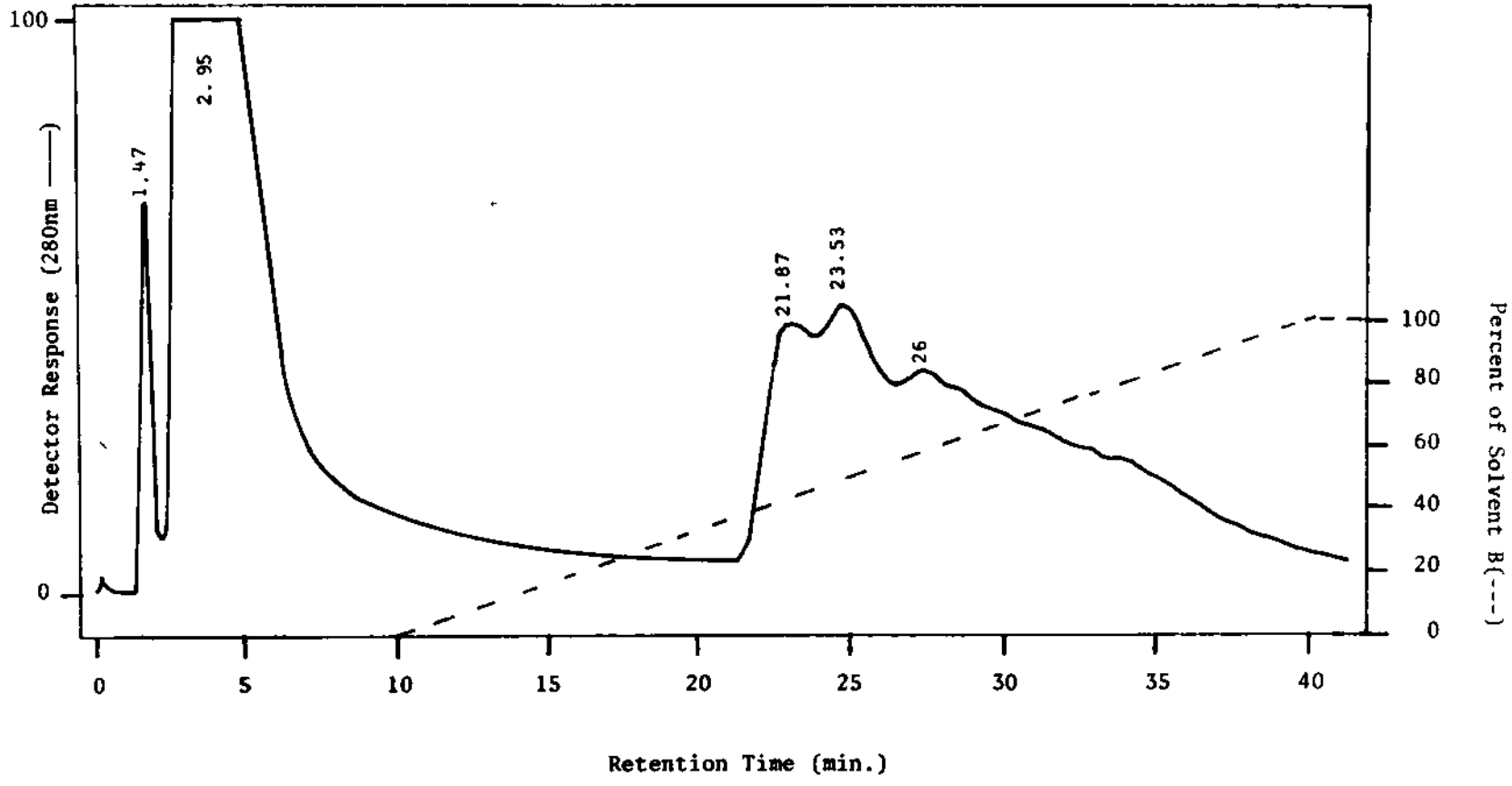

100
0
Detector Response (280nm ——)
1.47
2.95
21.87
23.53
26
100
80
60
40
20
0
Percent of Solvent B(---)
0
5
10
15
20
25
30
35
40
Retention Time (min.)

Fig. 9--Ion-exchange chromatography of histoplasmin (HKC-43) using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 Å with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

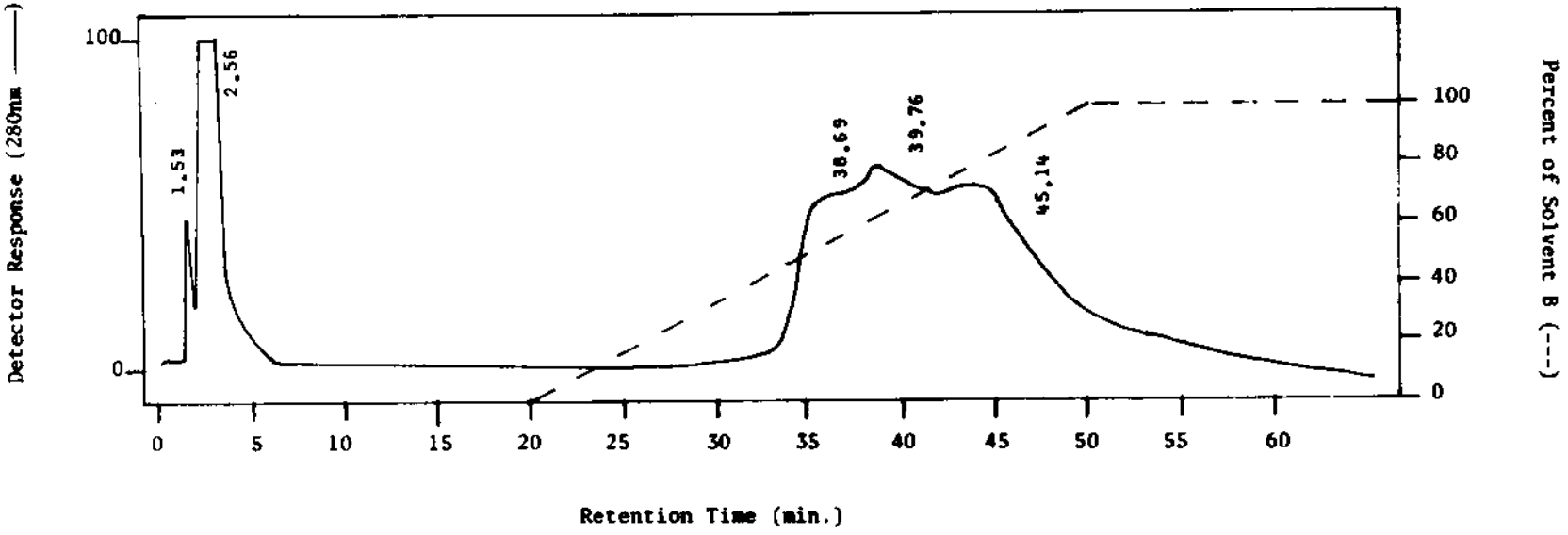
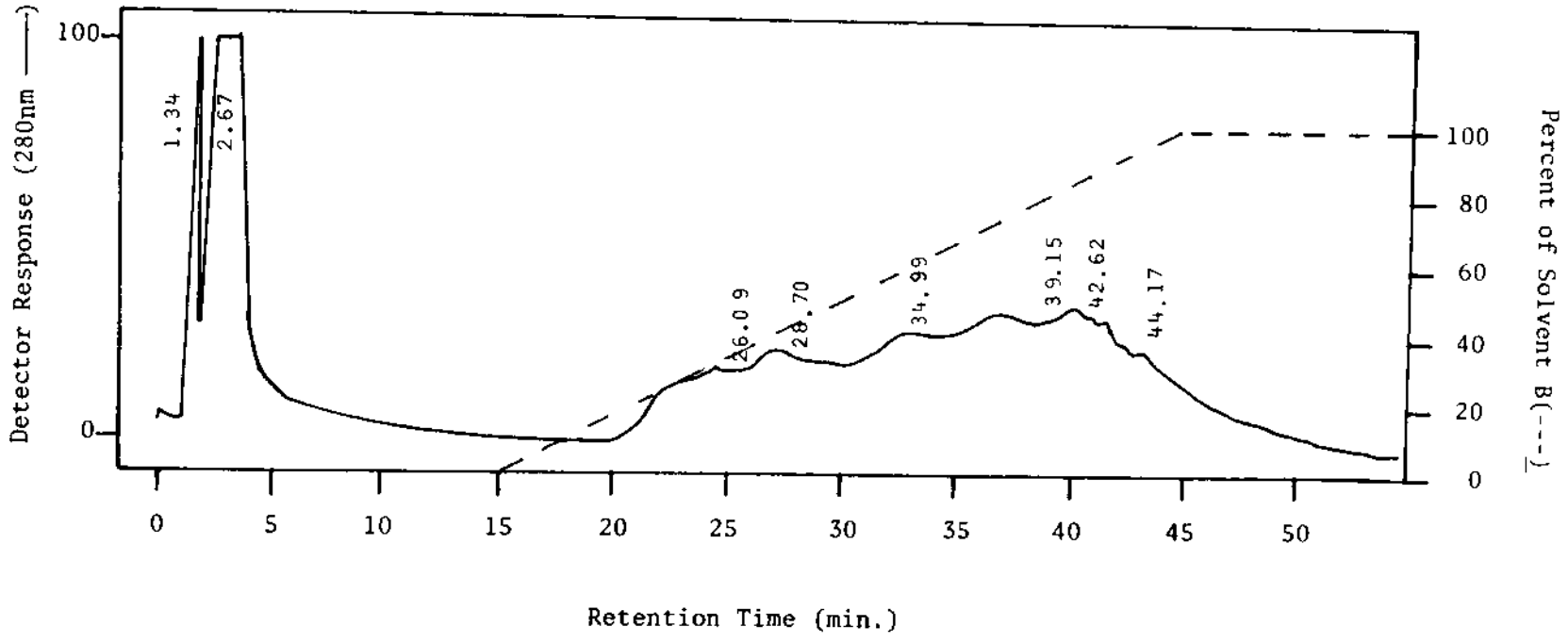

Detector Response (280nm ——)
100
0
1.53
2.56
38.69
39.76
45.14
0
5
10
15
20
25
30
35
40
45
50
55
60
Retention Time (min.)
100
80
60
40
20
0
Percent of Solvent B (---)

Fig. 10--Ion-exchange chromatography of blastomycin (KCB-26) using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 Å with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.


Detector Response (280nm ——)
100
0
1.34
2.67
26.09
28.70
34.99
39.15
42.62
44.17
0
5
10
15
20
25
30
35
40
45
50
Retention Time (min.)
100
80
60
40
20
0
Percent of Solvent B(---)

the previous two antigen preparations, baseline separation of two major peaks which were unbound to the column, but, more importantly, it shows the presence of no less than six smaller peaks that were bound to the column and hence required elution by the linear salt gradient. Figure 11 shows the ion-exchange chromatographic pattern obtained with b-ASWS. It is striking in that the ion-exchange pattern looks very close to that pattern (Figure 4) obtained with size exclusion chromatography. Again, baseline separation was obtained for two unbound peaks. Additionally, the bound peak at 15.01 minutes, because of its shape and relative area measurement, may correspond to the peak seen at 7.45 minutes on the size exclusion chromatographic pattern. As shown in Figure 12, the ion-exchange chromatographic pattern obtained with coccidioidin is still puzzling. Even though baseline separation was obtained for two components, the bulk of the constituents interacted ionically with the column but eluted in one large diffuse peak. In comparison to the SEC chromatogram (Figure 5), the ion-exchange chromatogram showed coccidioidin to be a more complex mixture. Still, the use of either SEC or ion-exchange chromatography appeared to be unsatisfactory for adequate separation of the components in this complex mixture. The ion-exchange chromatographic pattern shown in Figure 13 is that of c-ASWS. As with the previous five antigenic preparations, baseline separation was obtained for two unbound component peaks at the beginning of

Fig. 11--Ion-exchange chromatography of an alkali-soluble, water-soluble antigen of Blastomyces dermatitidis (b-ASWS) using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 Å with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

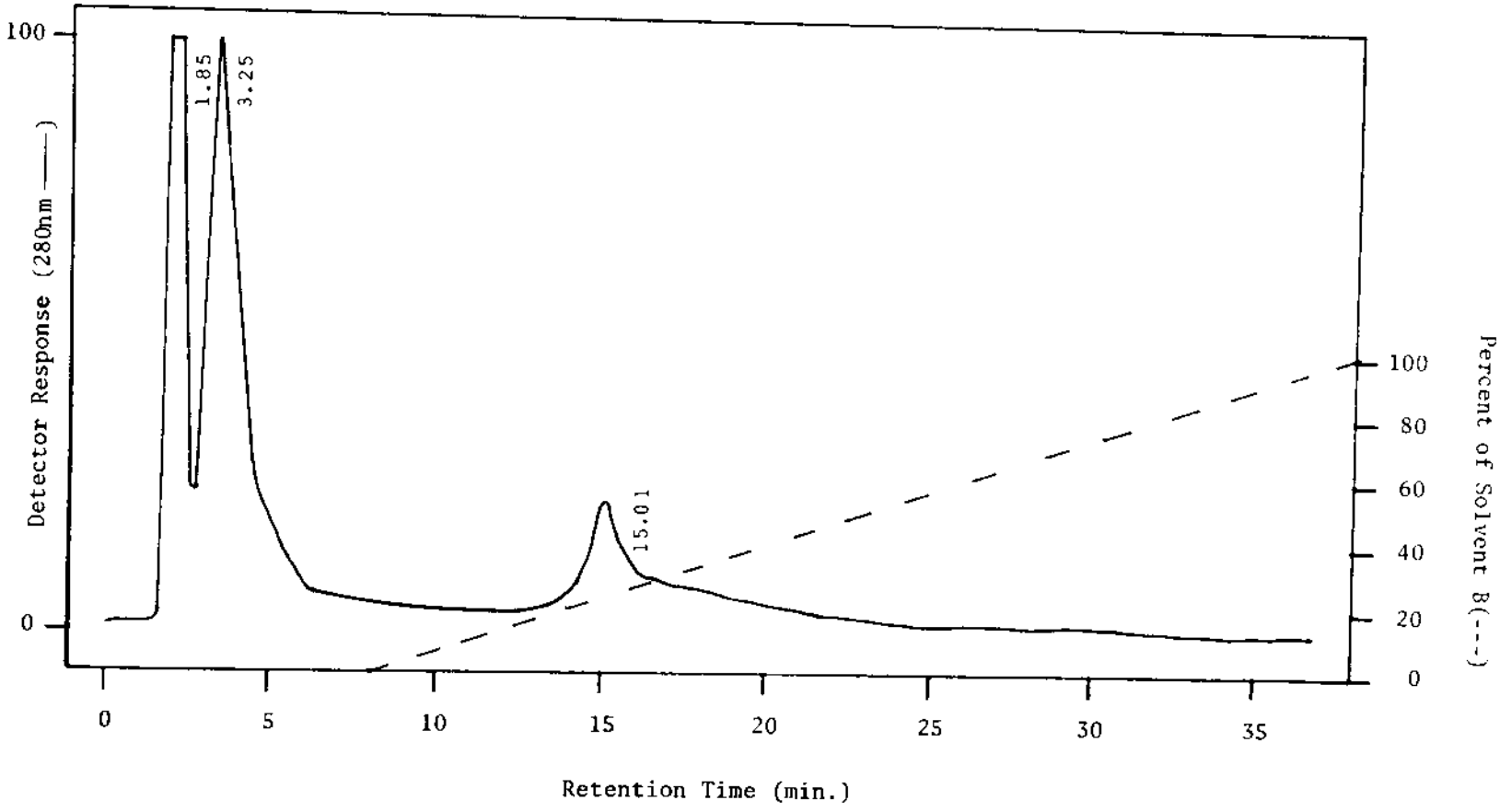

100
0
Detector Response (280nm ——)
1.85
3.25
15.01
0
5
10
15
20
25
30
35
Retention Time (min.)
100
80
60
40
20
0
Percent of Solvent B(---)

Fig. 12--Ion-exchange chromatography of coccidioidin using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 Å with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.

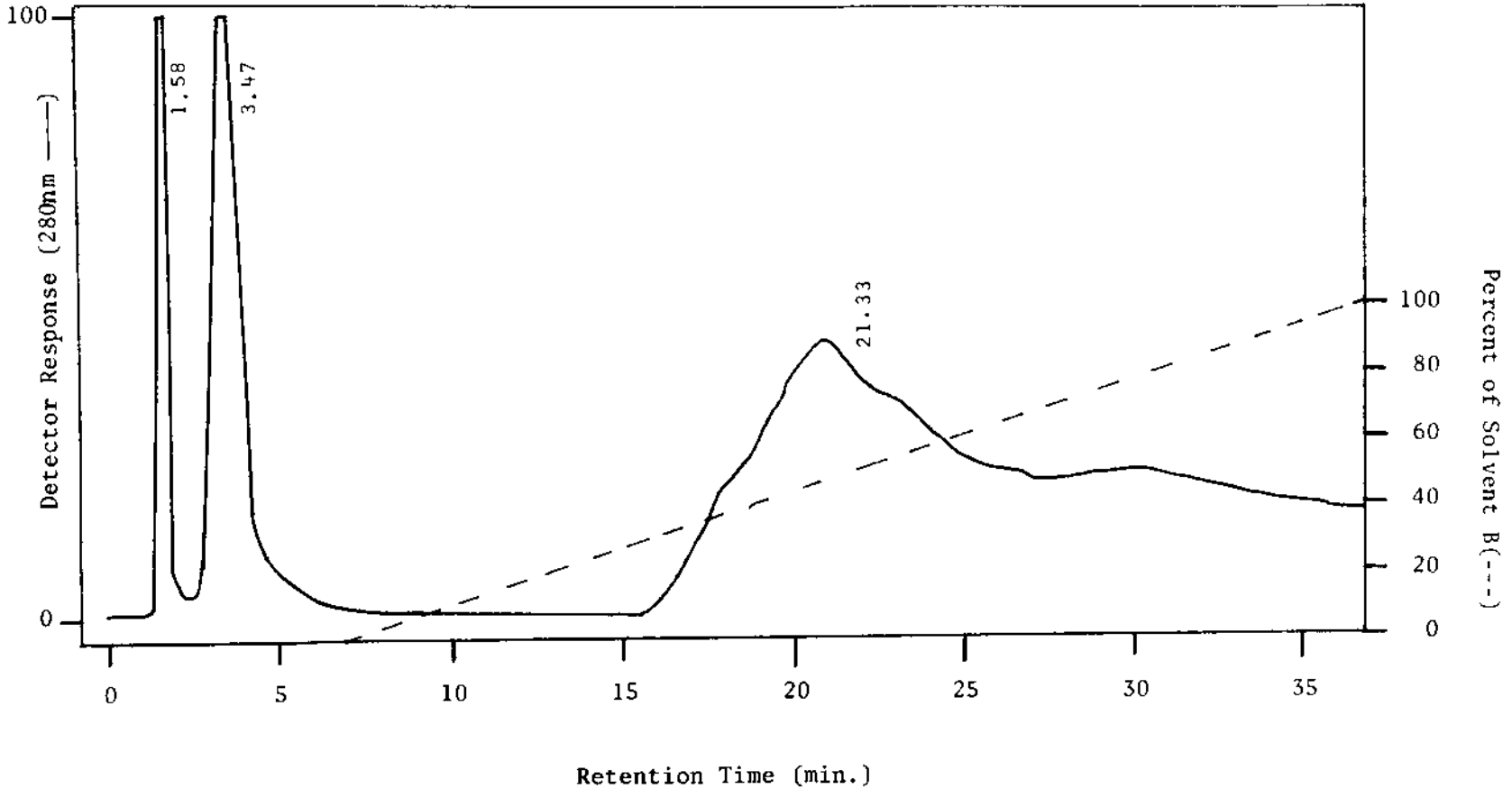
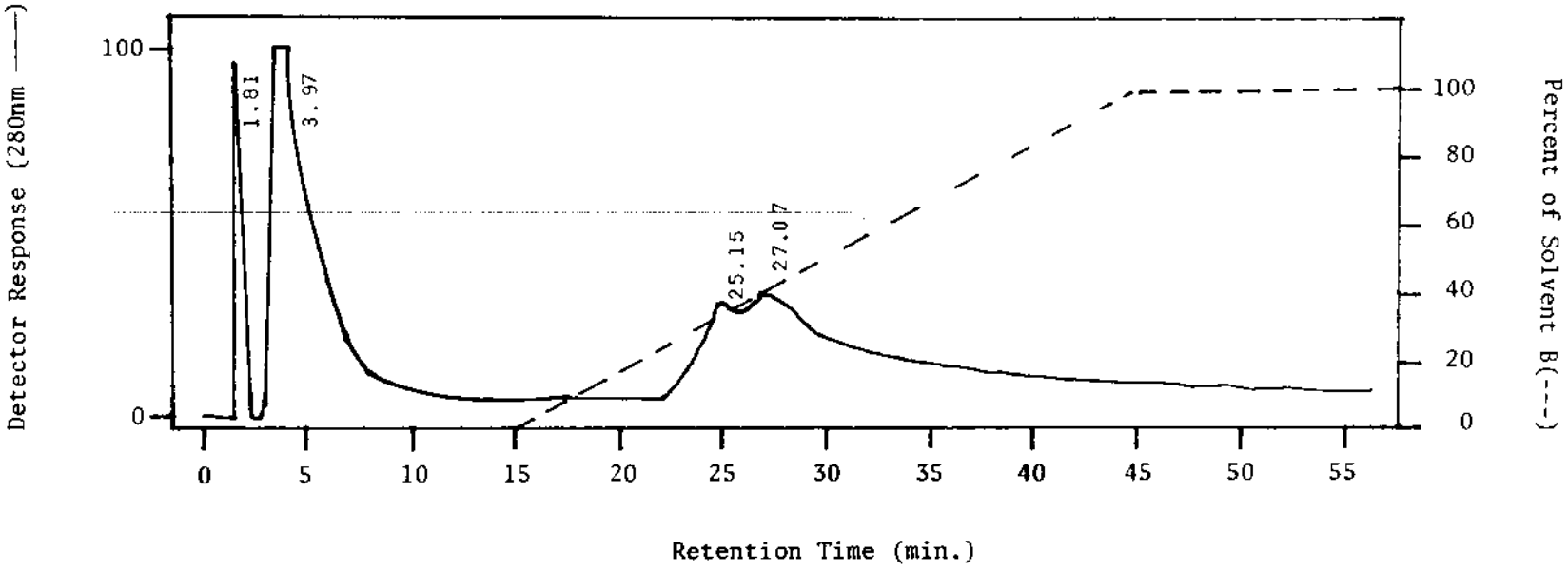

100
0
Detector Response (280nm ——)
1.58
3.47
21.33
100
80
60
40
20
0
Percent of Solvent B(- - -)
0
5
10
15
20
25
30
35
Retention Time (min.)

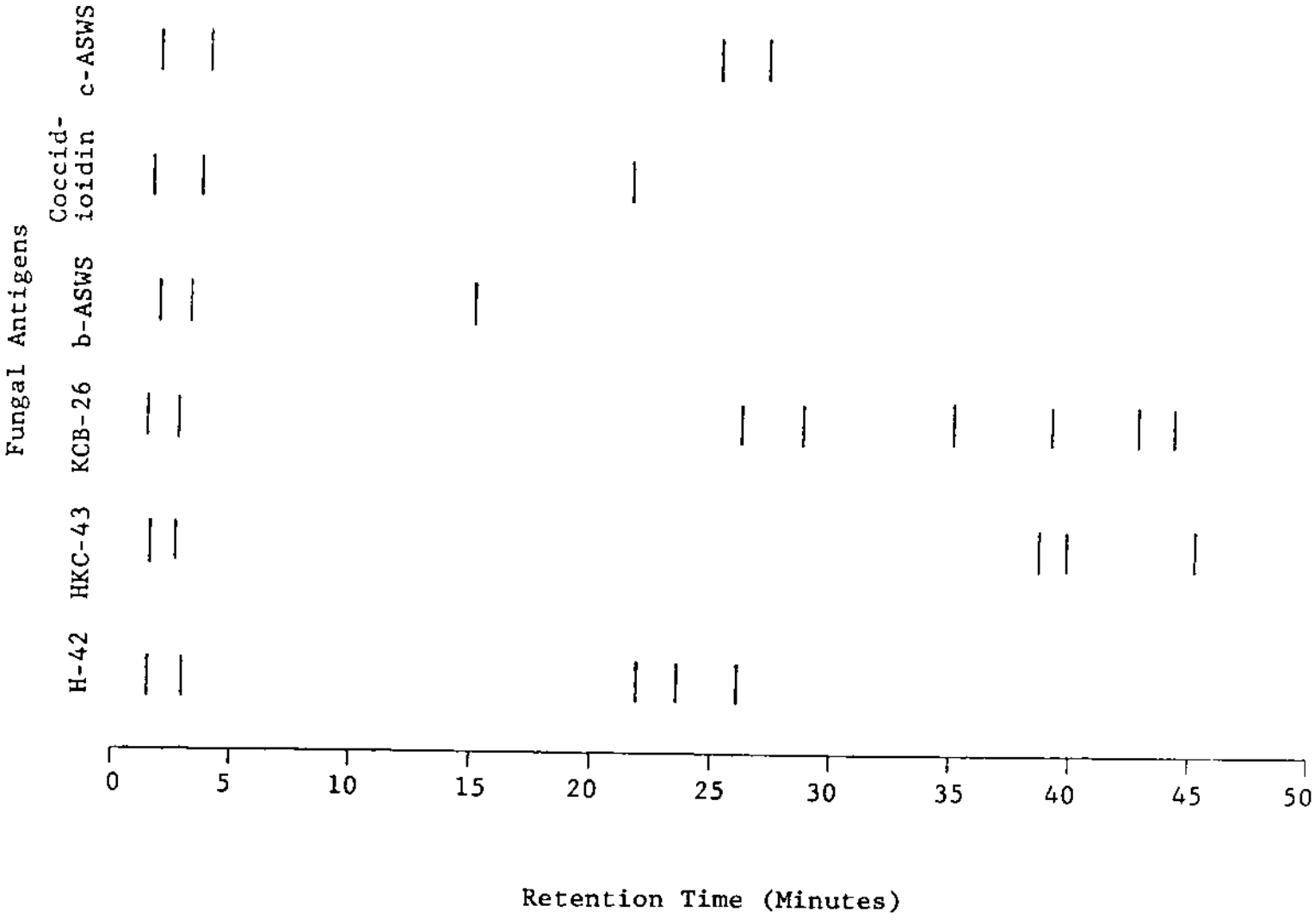
Fig. 13--Ion-exchange chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 Å with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.


Detector Response (280nm ——)
100
0
1.81
3.97
25.15
27.07
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min.)
100
80
60
40
20
0
Percent of Solvent B(---)

the chromatogram. In the linear gradient, or bound portion of the chromatogram, the pattern demonstrates two notable peaks. If this pattern is compared to the pattern that was shown in Figure 6, ion-exchange chromatography has demonstrated at least one more peak than was demonstrated by size exclusion chromatography. However, it is possible now that the two overlapping peaks shown in the size exclusion chromatographic pattern (Figure 6) have been separated at the beginning of the ion-exchange chromatographic pattern and that the third peak, retention time 5.71 minutes, has been separated into two peaks through interaction with the column.

Again, in order to compare the component retention times among the six different fungal antigen preparations, Figure 14 is a schematic representation consisting of vertical bars representing the various component peaks obtained in the ion-exchange chromatographic separation of the six fungal antigen preparations. Retention in ion-exchange chromatography was controlled by two independent phenomena: 1) the inherent size exclusion contribution from differential penetration by solutes of macroporous matrices, and 2) electrostatic partitioning at the surface of the ion-exchange. In the case of these six fungal antigen preparations, Figure 14 points out that they all contain two components (range 1.34-3.97) which are similar in terms of molecular weight if not in terms of their structure. Further, in the gradient portion of the chromatogram, each of the antigen preparations showed a unique component peak pattern.

Fig. 14--Schematic representation of the protein map chromatograms of fungal antigen preparations obtained by ion-exchange chromatography. The vertical bars represent the average retention times of component peaks of triplicate runs (±0.15 min) for each fungal antigen preparation.


Fungal Antigens
c-ASWS
Coccid-ioidin
b-ASWS
KCB-26
HKC-43
H-42
0
5
10
15
20
25
30
35
40
45
50
Retention Time (Minutes)

Figures 15 through 20 show chromatograms that were obtained when the six fungal antigen preparations were subjected to reverse-phase chromatography. Figure 15 shows the reverse-phase chromatographic pattern obtained from histoplasmin H-42. The first noticeable thing is a large hydrophilic peak and then a large number of hydrophobic peaks that occurred only with gradient separation. Figure 16 shows the reverse-phase chromatographic pattern of histoplasma HKC-43, and very much like that shown in Figure 15, the pattern shows a large hydrophilic peak and then a large group of peaks that are hydrophobic in nature. The pattern shown in Figure 17 is that of blastomycin KCB-26. Again, it shows a large hydrophilic peak and a large hydrophobic peak followed by multiple smaller component peaks all of which are also hydrophobic in nature. Figure 18 details the reverse-phase pattern obtained with b-ASWS. The pattern shown in Figure 18, at least in the hydrophilic portion of the chromatograms, is very similar to the pattern obtained when this antigen was subjected to size exclusion chromatography (Figure 4). The hydrophilic region demonstrates two major peaks followed by a much smaller third peak. This looks very similar to the pattern obtained with the size exclusion chromatographic analysis of this particular antigen. Unlike the preceding three reverse-phase chromatographic patterns, the separation in the hydrophobic region of this chromatogram is not as dramatic. Figure 19 shows the reverse-phase pattern obtained

Fig. 15--Reverse-phase chromatography of histoplasmin (H-42) using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.

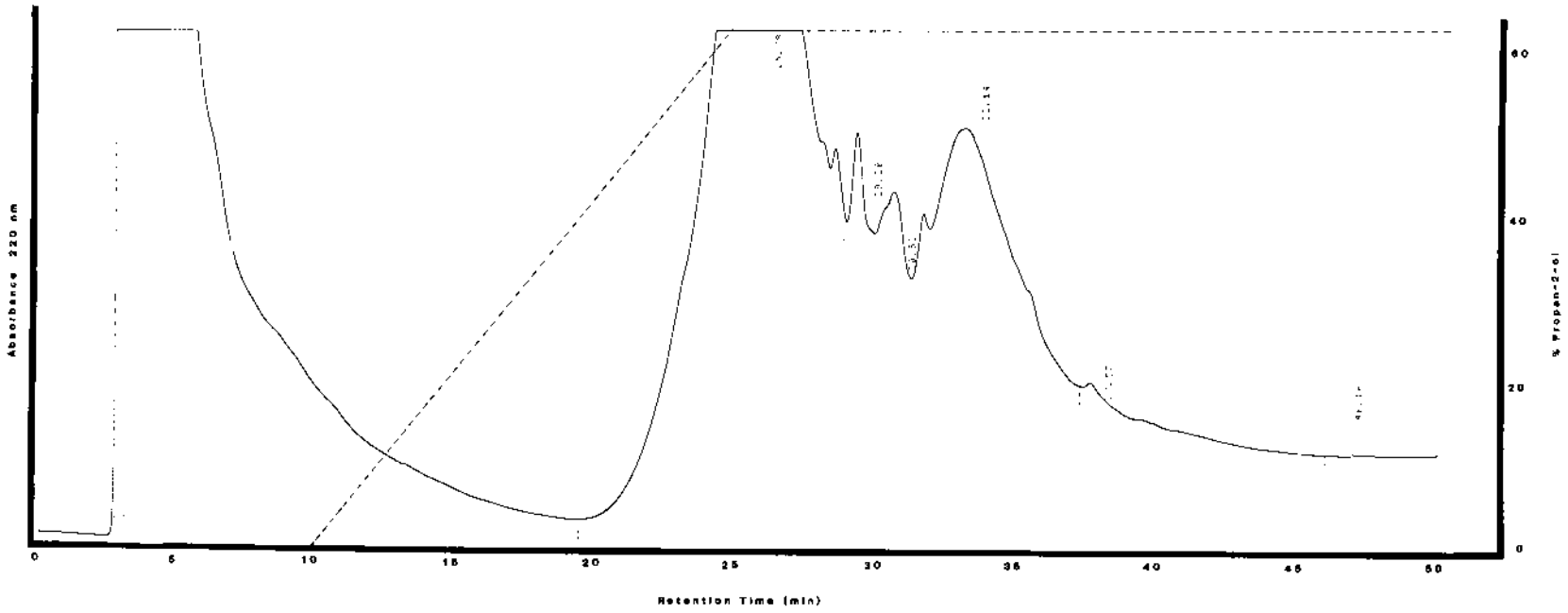

Absorbance 220 nm
0
5
10
15
20
25
30
35
40
45
50
Retention Time (min)
0
20
40
60
% Propan-2-ol

Fig. 16--Reverse-phase chromatography of histoplasmin (HKC-43) using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.

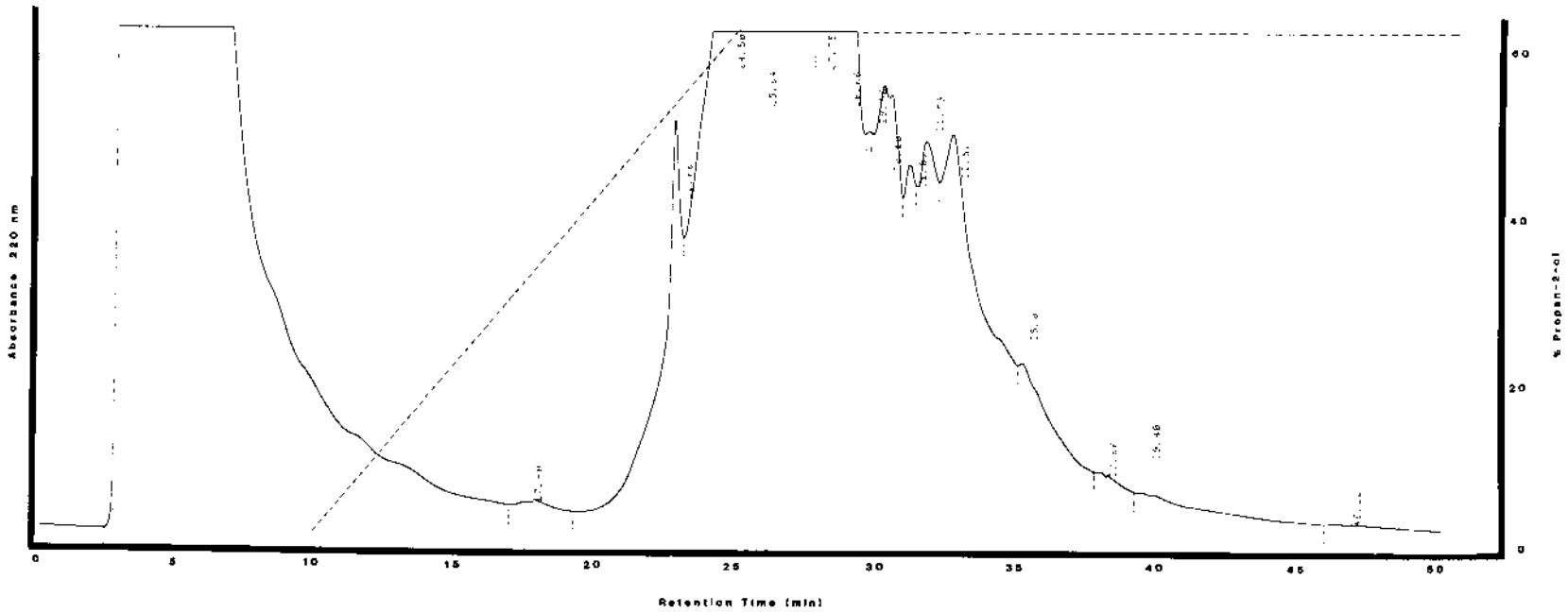

Absorbance 220 nm
0
5
10
15
20
25
30
35
40
45
50
Retention Time (min)
0
20
40
% Propan-2-ol

Fig. 17--Reverse-phase chromatography of blastomycin (KCB-26) using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.

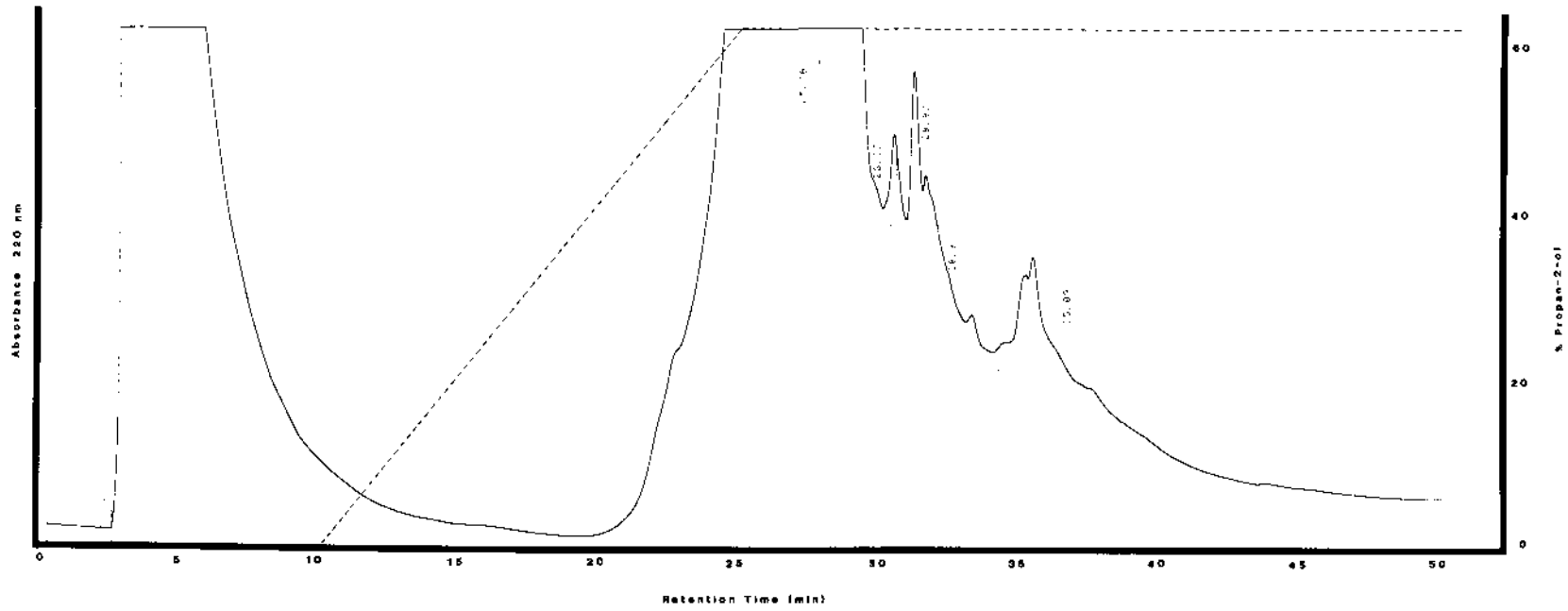

Absorbance 220 nm
0
5
10
15
20
25
30
35
40
45
50
Retention Time (min)
0
20
40
60
% Propan-2-ol

Fig. 18--Reverse-phase chromatography of an alkali-soluble, water-soluble antigen of Blastomyces dermatitidis (b-ASWS) using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.

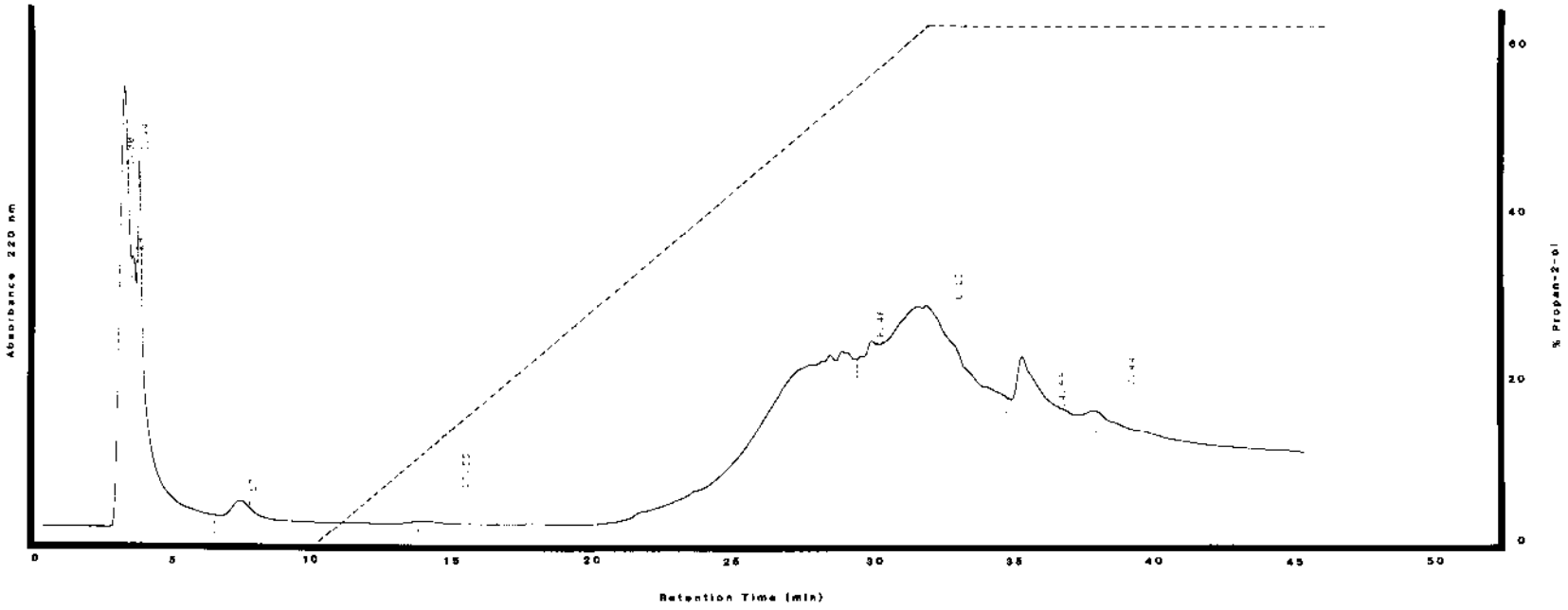
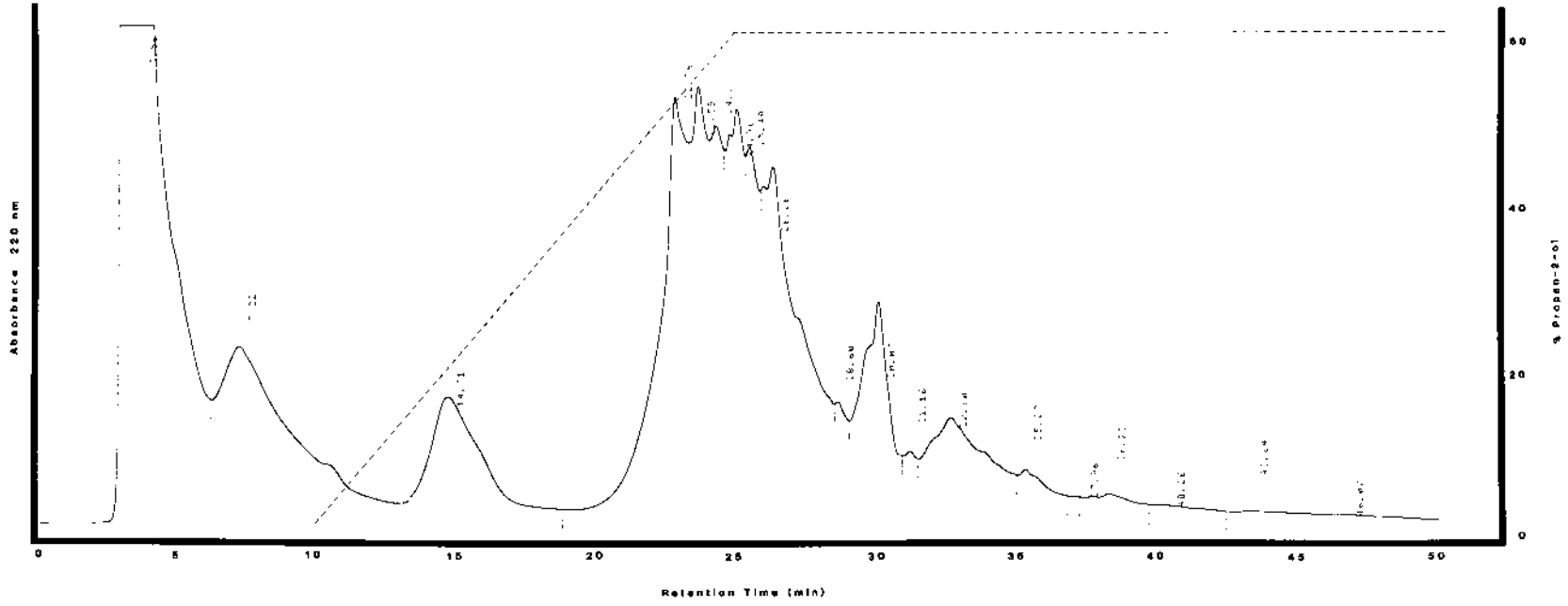

Absorbance 220 nm
% Propan-2-ol
Retention Time (min)
0
5
10
15
20
25
30
35
40
45
50
0
20
40
60

Fig. 19--Reverse-phase chromatography of coccidioidin using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.


Absorbance 220 nm
% Propan-2-ol
Retention Time (min)
0
5
10
15
20
25
30
35
40
45
50
80
40
20
0

with coccidioidin, and like its predecessor patterns, it showed baseline separation in the hydrophilic region of the chromatogram. Also, this chromatogram demonstrates the presumed complex native of this antigen preparation. The gradient portion of the reverse-phase chromatogram contains multiple, distinct component peaks. Figure 20 shows the reverse-phase chromatographic separation of c-ASWS. Like the pattern shown in Figure 18 (b-ASWS), the hydrophilic portion of this chromatogram was very similar to the pattern demonstrated when it was subjected to size exclusion chromatography (Figure 6). Unlike Figure 19, the pattern demonstrated in the hydrophobic region of the chromatogram is relatively simple and shows, in some instances, good resolution between component peaks.

Figure 21 is a schematic representation of the protein map chromatograms of the six fungal antigen preparations obtained with reverse-phase chromatography. As with the previous two schematics (Figures 7 and 14), this representation shows that the six fungal antigens do contain components that are similar. However, each fingerprint of each preparation is unique. Thus, reverse-phase chromatographic analysis appears to be the method most useful for fingerprinting and separating fungal antigens. The separated components could then be tested immunologically to determine if components with similar retention times are the cause of the cross-reactivity seen with the fungal antigen preparations.

Fig. 20--Reverse-phase chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.

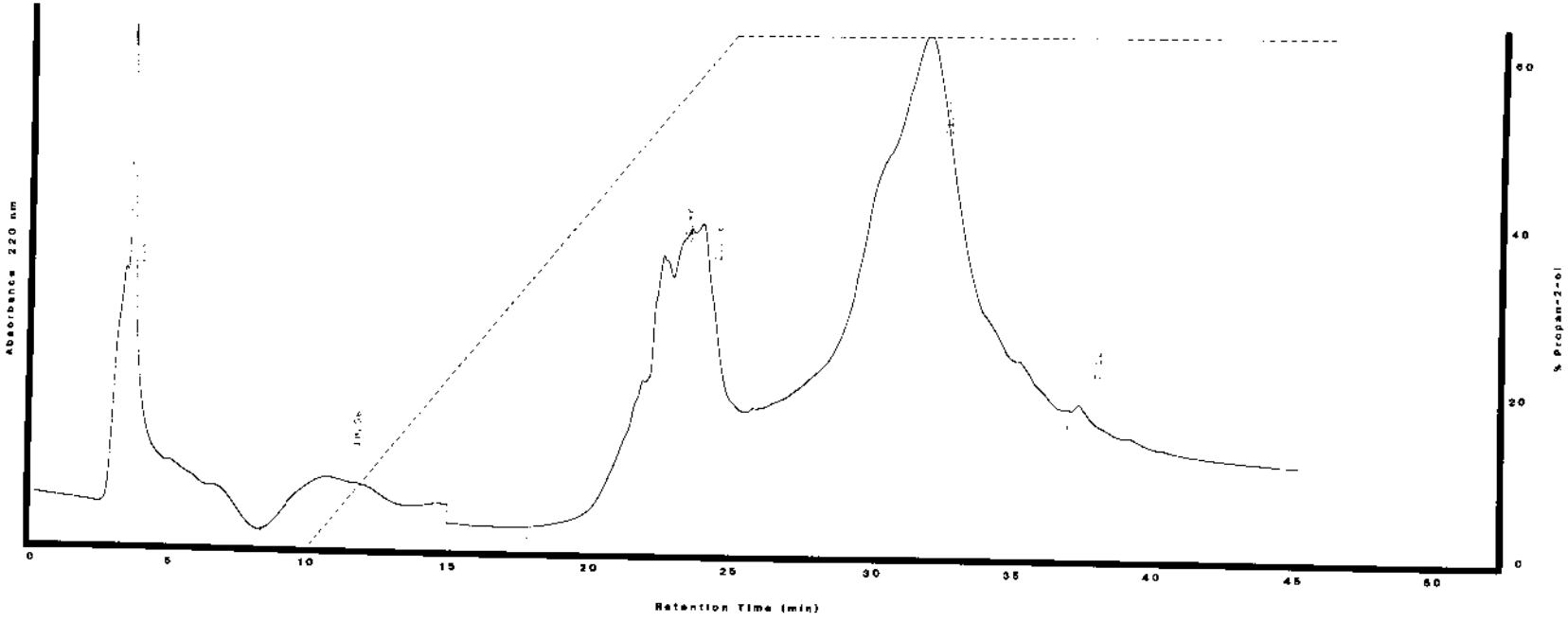
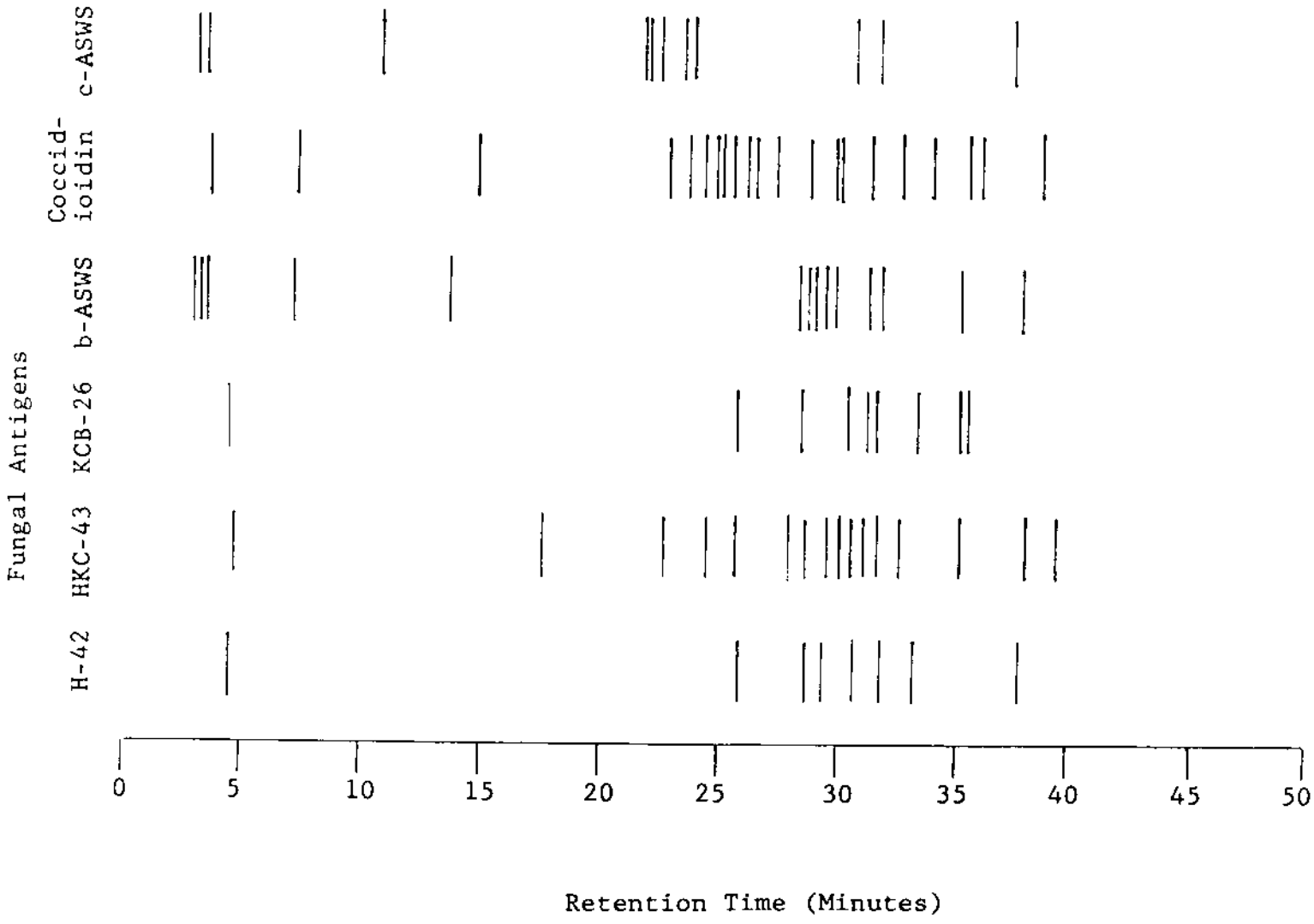

Absorbance 220 nm
0
5
10
15
20
25
30
35
40
45
50
Retention Time (min)
60
40
20
0
% Propan-2-ol

Fig. 21--Schematic representation of the protein map chromatograms of fungal antigen preparations obtained by reverse-phase chromatography. The vertical bars represent the average retention times of protein peaks of triplicate runs (±0.15 min) for each fungal antigen preparation.


Fungal Antigens
c-ASWS
Coccid-ioidin
b-ASWS
KCB-26
HKC-43
H-42
0
5
10
15
20
25
30
35
40
45
50
Retention Time (Minutes)

Preliminary Structural Characterization of an Alkali-Soluble, Water-Soluble Antigen of Coccidioides immitis

Table I details the main bands that occur on infra-red spectrographic analysis of an alkali-soluble, water-soluble antigen (c-ASWS) obtained from Coccidioides immitis. The table shows that the main components of the antigen, as demonstrated by infra-red spectroscopy, are mannans and protein. The band at 3350 cm^{-1} indicates the presence of hydroxyl groups in the antigen. The band at 2925 cm^{-1} has been shown (85) to be characteristic of both yeast mannans and glucans and has been used to distinguish these compounds from cellulose which lacks a strong band at or near 2925 cm^{-1}. The bands at both 1640 and 1530 cm^{-1} suggest the presence of protein. The bands at 1400 and 1385 have been previously shown to be present in other reference mannans. Finally, the band at 812 cm^{-1} has been shown to be characteristic of both alpha and beta-linked mannans.

Table II shows the primary amino acid analysis of c-ASWS. Aspartic acid and glutamic acid were found to account for 27% of the peptide material in the c-ASWS. The other 13 amino acids found in c-ASWS account for the remaining 73% of the peptide structure. Proline, tryptophan, and cysteine were not detected in this primary analysis. This result is unusual since these amino acids play a major role both in the secondary and tertiary structure make-up of the protein core of a glycoprotein.

TABLE I

INFRA-RED SPECTRUM OF c-ASWS

Frequency (cm^{-1})	Relative Intensity*	Significance
3350	S	Hydroxyl groups
2925	M	Characteristic of yeast mannans and glucans
1640	M	Mono-substituted amide group; c=o stretching
1530	M	N-H deformation mode; acetamido groups
1400	W	Found in all
1385	W	mannans
1300-1250	W	Phosphate esters
1050	S	Found in fungal cell wall
978	W	polymers
812	W	Characteristic of both α- and β-mannans

*S = strong; M = moderate; W = weak

TABLE II

AMINO ACID ANALYSIS OF c-ASWS

Amino Acid	Micrograms	Micromoles	Weight Percent in Peptide
Aspartic Acid	32.46	0.282	12.26
Threonine	16.74	.166	6.33
Serine	16.72	.192	6.32
Glutamic Acid	39.04	.302	14.75
Glycine	14.74	.258	5.57
Alanine	17.52	.246	6.62
Valine	17.52	.176	6.62
Methionine	5.74	.044	2.17
Isoleucine	14.40	.128	5.44
Leucine	19.20	.170	7.25
Tyrosine	10.18	.062	3.85
Phenylalanine	11.66	.080	4.41
Histidine	12.84	.094	4.85
Lysine	19.28	.150	7.28
Arginine	16.62	.106	6.28
Total	264.66	2.46	100

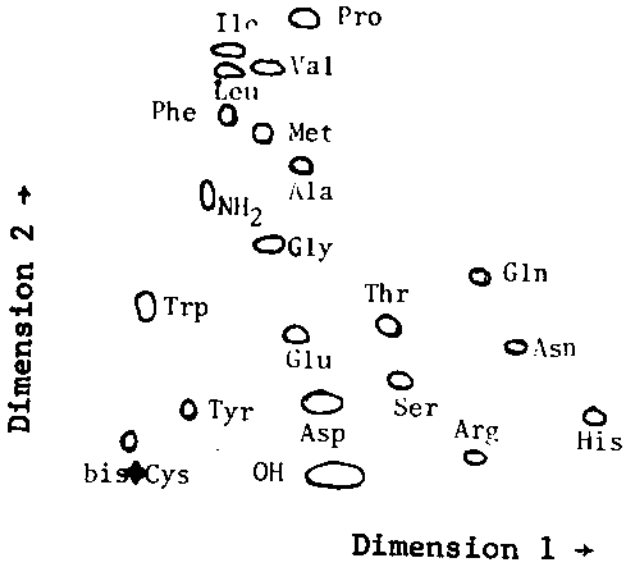
Figure 22 shows diagrammatically the results from an N-terminal analysis of c-ASWS using dansyl chloride (DNS). This analysis revealed the presence of eight dansyl amino acids. These may represent N-terminals from eight different proteins or simply free amino acids. Further work is necessary to determine which of these two possibilities is correct.

Table III shows the results of the application of the alkaline beta-elimination reduction reactions on c-ASWS. All of the threonine residues have been shown by this technique to be involved in 0-glycosidic linkages to carbohydrate chains. For serine, unlike threonine, only half of the available serine residues have been shown to be involved in 0-glycosidic linkages with carbohydrates.

Table IV continues detailing the chemical properties of c-ASWS. The monosaccharide content was found to be 55% with the molar ratios of 3-0-methyl mannose:mannose:galactose were 1:7.6:1.6. Peptide was found to be present at a level of 26%. The lipid content was determined to be 4.2%, as determined by extraction and gravimetric analysis.

The antigen was also found to be reactive with concanavalin A by immunodiffusion. A single, well defined precipitin band was seen on the immunodiffusion plate. In addition to being a single distinct band, the band was also quite sharp and not diffuse as would have been expected from a polydisperse preparation.

Fig. 22--Diagramatic two-dimensional separation of common DNS-amino acids on polyamide. Bottom-Diagramatic two-dimensional separation of the DNS-amino acids of an N-terminal analysis of c-ASWS. Chromatographic solvents: Dimension 1, water-pyridine-formic acid (93:3.5:3.5,v/v); Dimension 2, benzene-acetic acid (4.5:1,v/v).


Ile
Pro
Val
Leu
Phe
Met
Ala
NH_2
Gly
Gln
Thr
Trp
Glu
Asn
Ser
Tyr
Asp
Arg
His
bis
Cys
OH
Dimension 2 →
Dimension 1 →

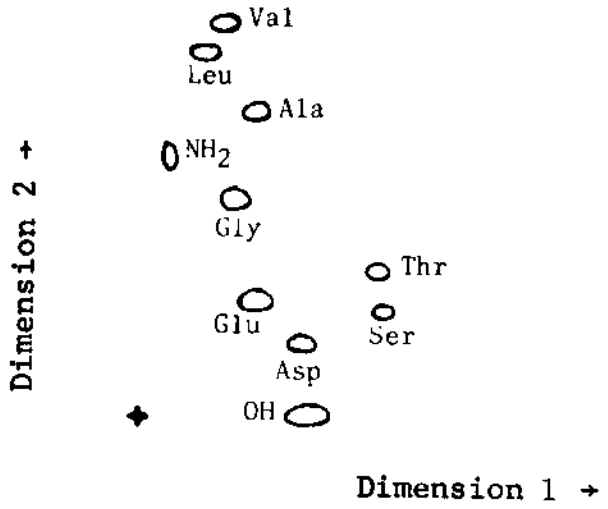

Val
Leu
Ala
NH_2
Gly
Thr
Glu
Ser
Asp
OH
Dimension 2 →
Dimension 1 →

TABLE III

ALKALINE β-ELIMINATION OF c-ASWS IN AQUEOUS SODIUM BOROHYDRIDE AND PALLADIUM CHLORIDE*

Amino Acid	Micrograms Left After β-Elimination and Reduction For Ten Hours
Threonine	
Loss of Threonine	6.3
Recovered as ABA**	6.09
Recovery, percent	96.7
Serine	
Loss of Serine	2.62
Recovered as Alanine	2.66
Recovery, percent	101.5

* Solutions (5 mg/ml) of c-ASWS in aqueous 0.1 M sodium borohydride were incubated at 45° C for ten hours. This treatment was followed by simultaneous additions of 0.66 M $NaBH_4$ and 0.016 M $PdCl_2$ in 0.8 M hydrochloric acid.

** ABA = -aminobutyric acid.

TABLE IV

PROPERTIES OF AN ALKALI-SOLUBLE, WATER-SOLUBLE CELL WALL ANTIGEN (c-ASWS) OF *COCCIDIOIDES IMMITIS*

Property	c-ASWS
Carbohydrate, Percent	55
Peptide, Percent	26
Lipid, Percent	4.2
Molecular Ratios:	
3-0-Methyl Mannose	1
Mannose	7.6
Galactose	1.6
Con A Affinity	Reactive
Molecular Weight	Peaks 1,2 - $>2 \times 10^5$
	Peak 3 - 59,485
	Peak 4 - 32,000

When c-ASWS was reduced using 6 M guanidine hydrochloride and 2-mercapoethanol, chromatography on a column of 4% cross-linked agarose (Figure 23) revealed four peaks containing both carbohydrate and protein. The peaks occurred in groups of two which were similar in molecular weight. The first component pair co-eluted in the region of Blue Dextran. A molecular weight was not determined since this area corresponds to the void volume of the column. Thus, the two peaks must have a molecular weight greater than 200,000. The second two peaks were determined to have molecular weights of 59,485 and 32,000, respectively. These molecular weights were derived from the equation $M^{1/3} = (0.79141-F(v))/.0101086$ as detailed in the Appendix.

Figure 24 shows a representation of the chromatogram obtained when c-ASWS was subjected to size exclusion chromatography on a column of 500 Å pore size glycerol coated control pore glass. This chromatogram shows two large peaks, with fairly good separation, that contain both protein and carbohydrate as determined by uv absorption and refractive index, respectively. Further, analysis by size exclusion chromatography (Figure 25) using a 300 Å pore size column revealed two peaks of approximately the same size and a third peak that had baseline separation from the other two peaks. In Figure 26, the ion-exchange chromatography pattern of c-ASWS is shown, and it reveals a pattern similar to the

Fig. 23--Elution diagram of reduced c-ASWS on 4 percent agarose in 6 M guanidine hydrochloride. _____, A_{280}; -----, A_{490}.

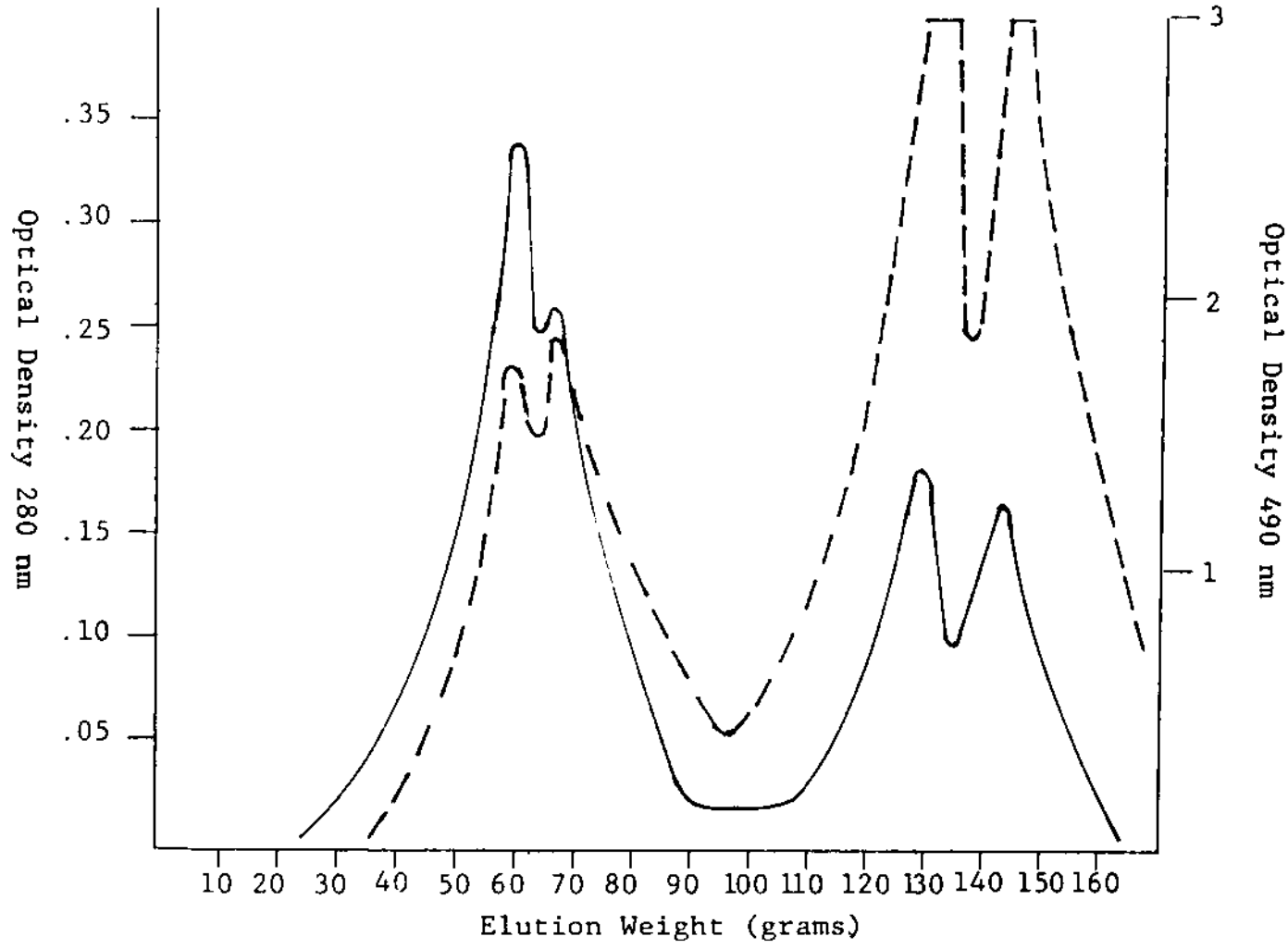

Optical Density 280 nm
.35
.30
.25
.20
.15
.10
.05
Optical Density 490 nm
3
2
1
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
Elution Weight (grams)

Fig. 24--Size exclusion chromatography of c-ASWS on a controlled-pore glass column using 0.015 M tris-acetate, pH 8.0; _____, uv, -----, RI.

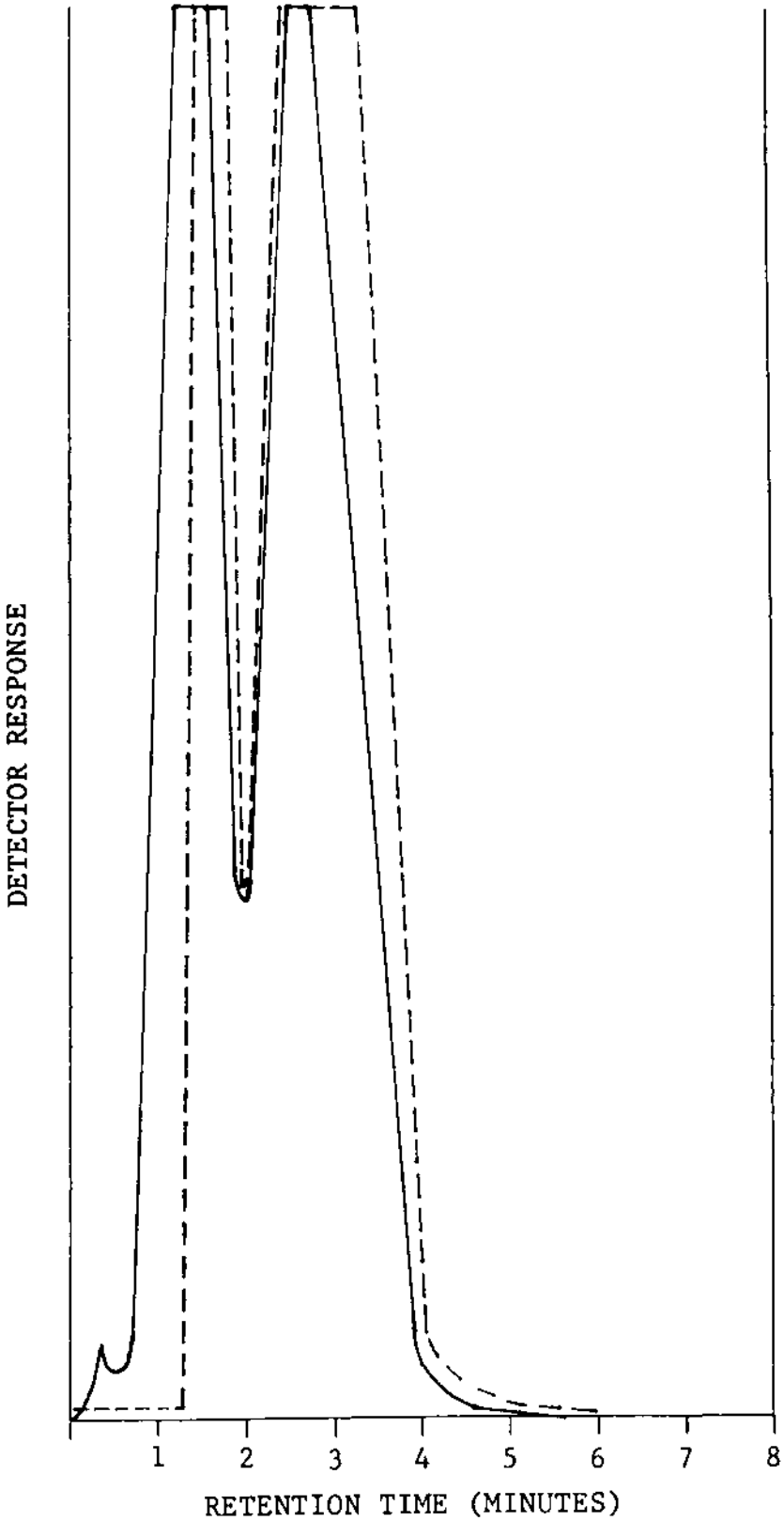

DETECTOR RESPONSE
1
2
3
4
5
6
7
8
RETENTION TIME (MINUTES)

Fig. 25--Size exclusion chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using 2.5 mM ammonium borate, pH 8.0 for the mobile phase; column, Synchropak 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed, 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detection, uv, 280 nm, 1.0 AUFS.

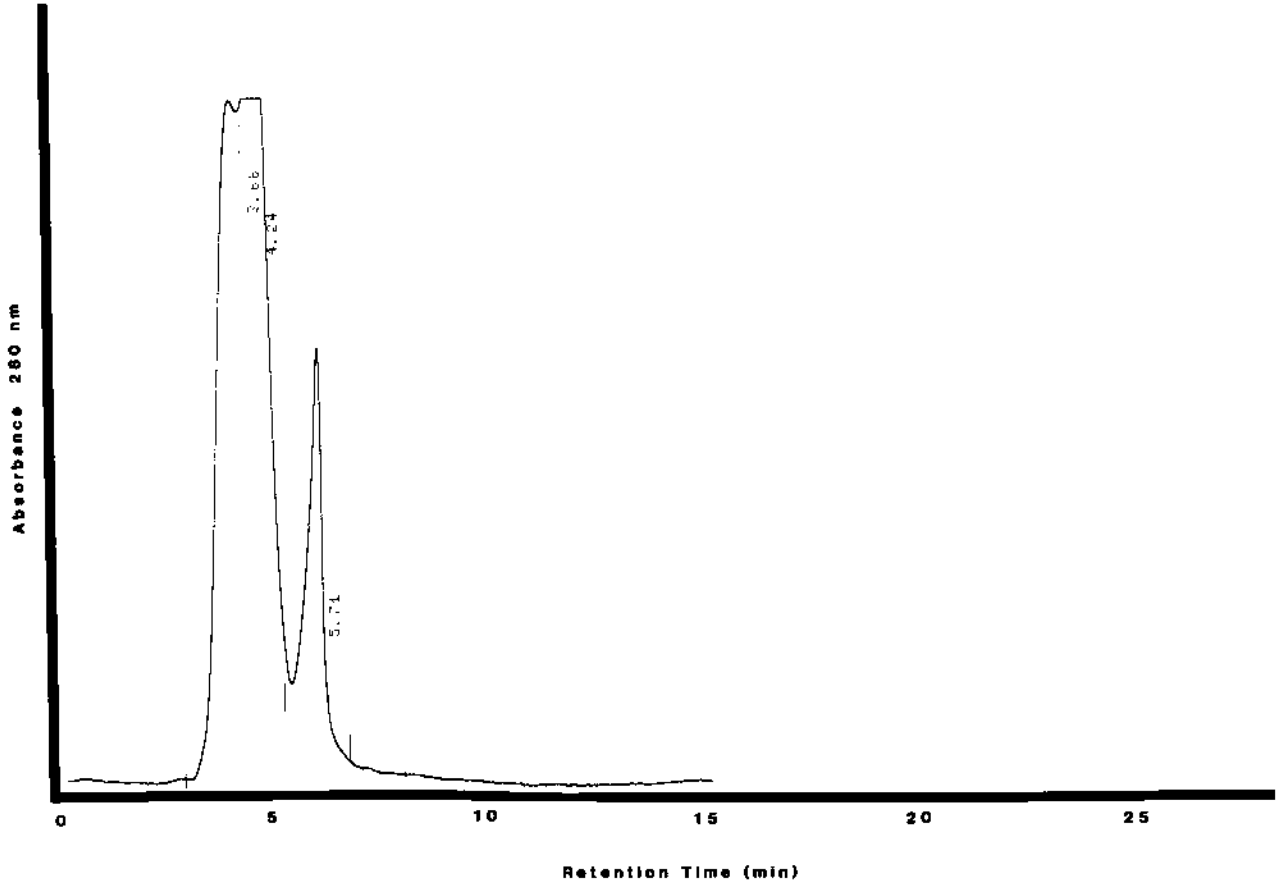
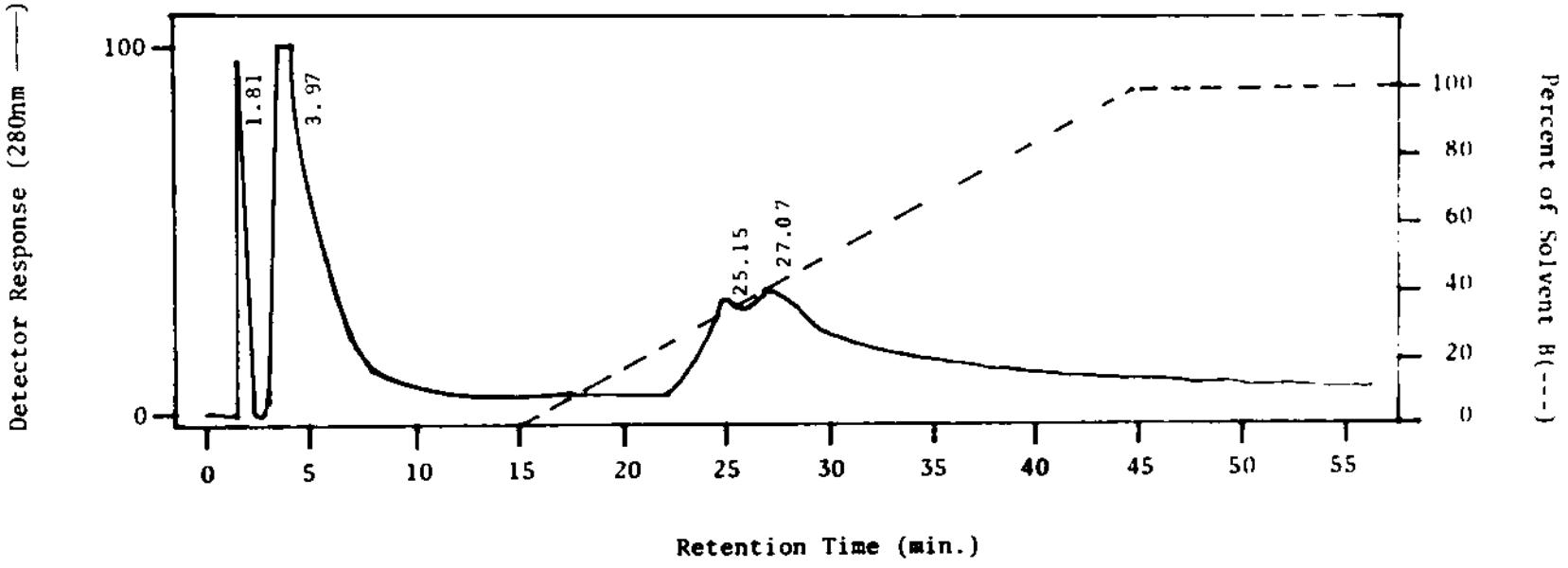

Absorbance 280 nm
3.66
4.24
5.71
0
5
10
15
20
25
Retention Time (min)

Fig. 26--Ion-exchange chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using 0.02 M tris-acetate, pH 7.9 as solvent A and 0.02 M tris-acetate, 0.4 M sodium acetate, pH 7.9 as solvent B; a 30 minute linear gradient was used for elution; column, a stainless-steel HPLC column (4.1 mm i.d. x 25 cm) was dry-packed with Electro-Nucleonics diethylaminoethyl-CPG, 170 A with a guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; chart speed 1 cm/min; injection volume, 100 µl; concentration 2.5 mg/ml; detector, uv, 280 nm, 1.0 AUFS.


Detector Response (280nm ——)
100
0
1.81
3.97
25.15
27.07
0
5
10
15
20
25
30
35
40
45
50
55
Retention Time (min.)
100
80
60
40
20
0
Percent of Solvent B(---)

pattern shown in Figure 25 in that we have two fairly large peaks which, although separated at the baseline, are probably close in their relative molecular weights, and then, two peaks that were bound to the column for which a separation is poor but again might indicate that the peaks are close both in molecular weight and probably in structure of the protein and/or carbohydrate subunits.

Reverse-phase chromatography (Figure 27) on a large pore (300 Å) reverse-phase column revealed two overlapping hydrophilic peaks and a smaller peak that was well separated from the first two larger peaks. A linear gradient with 2-propanol revealed a group of four overlapping peaks which were separated from one very large and a very small component peak. This separation further revealed that these peaks were very hydrophobic in nature. Finally, Figure 28 shows the separation that was obtained upon the use of a bonded primary amine column. Two large well separated carbohydrate containing peaks as demonstrated by absorbance at 195 nm, were obtained. A group of four smaller and possibly overlapping peaks occurred in between the large well separated carbohydrate containing peaks.

Gas-Liquid Chromatographic Analysis of Human Serum Samples

Table V lists the type and number of human serum samples that were analyzed by gas-liquid chromatography (GLC). The

Fig. 27--Reverse-phase chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using 0.013 M (0.1% v/v) TFA in water as the primary mobile phase and 2-propanol as the secondary phase; elution was carried out with a 60 minute linear gradient from 0 to 60% of 2-propanol in 0.1% (v/v) TFA; column, Synchropak RP-P, 300 Å (4.1 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); flow rate, 0.5 ml/min; injection volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 220 nm, 1.28 AUFS.

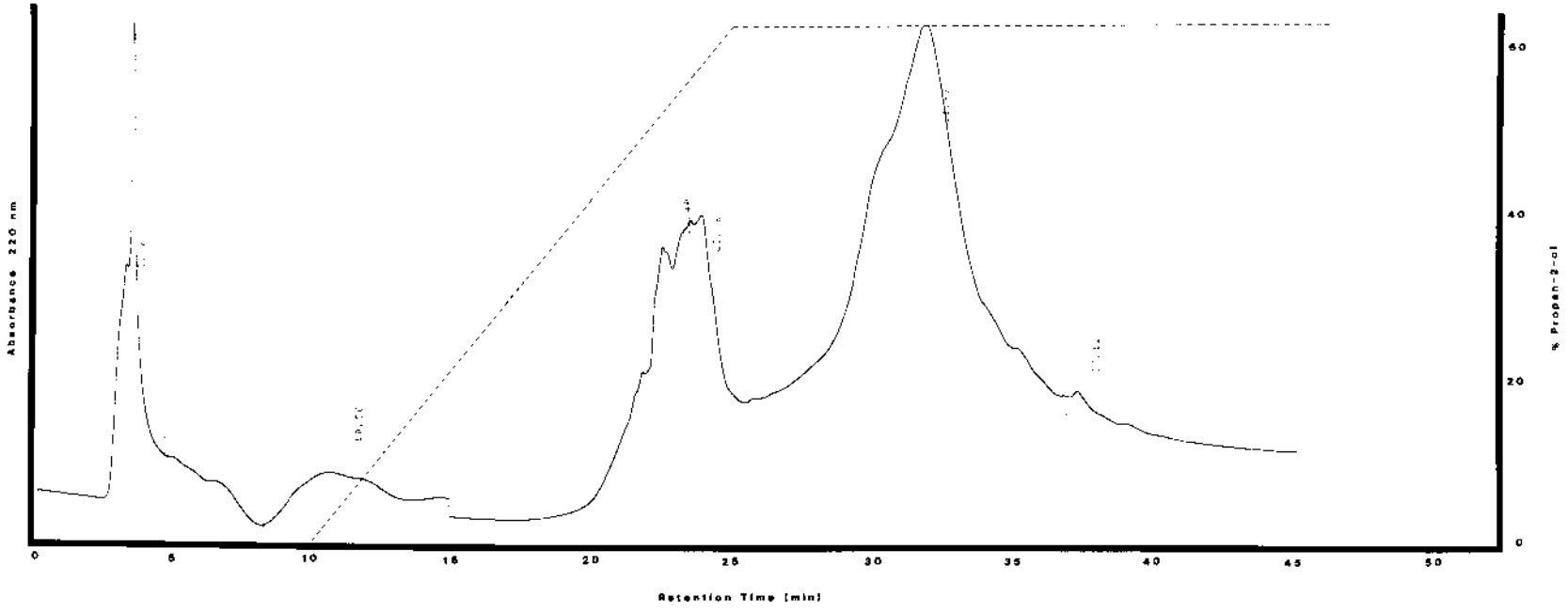

Absorbance 220 nm
0
5
10
15
20
25
30
35
40
45
50
Retention Time [min]
0
20
40
60
% Propan-2-ol

Fig. 28--High-performance, liquid chromatography of an alkali-soluble, water-soluble antigen of Coccidioides immitis (c-ASWS) using a bonded-primary amine column; column, Spherisorb-NH_2, 5 cm (4.6 mm i.d. x 25 cm) with guard column (4.6 mm i.d. x 4 cm); elution was performed with a linear gradient of 17:3 to 3:2 (v/v) acetonitrile-water for 20 min; flow, 1 ml/min; chart speed, 1 cm/min; injector volume, 100 µl; concentration, 2.5 mg/ml; detector, uv, 195 nm, 1.28 AUFS.

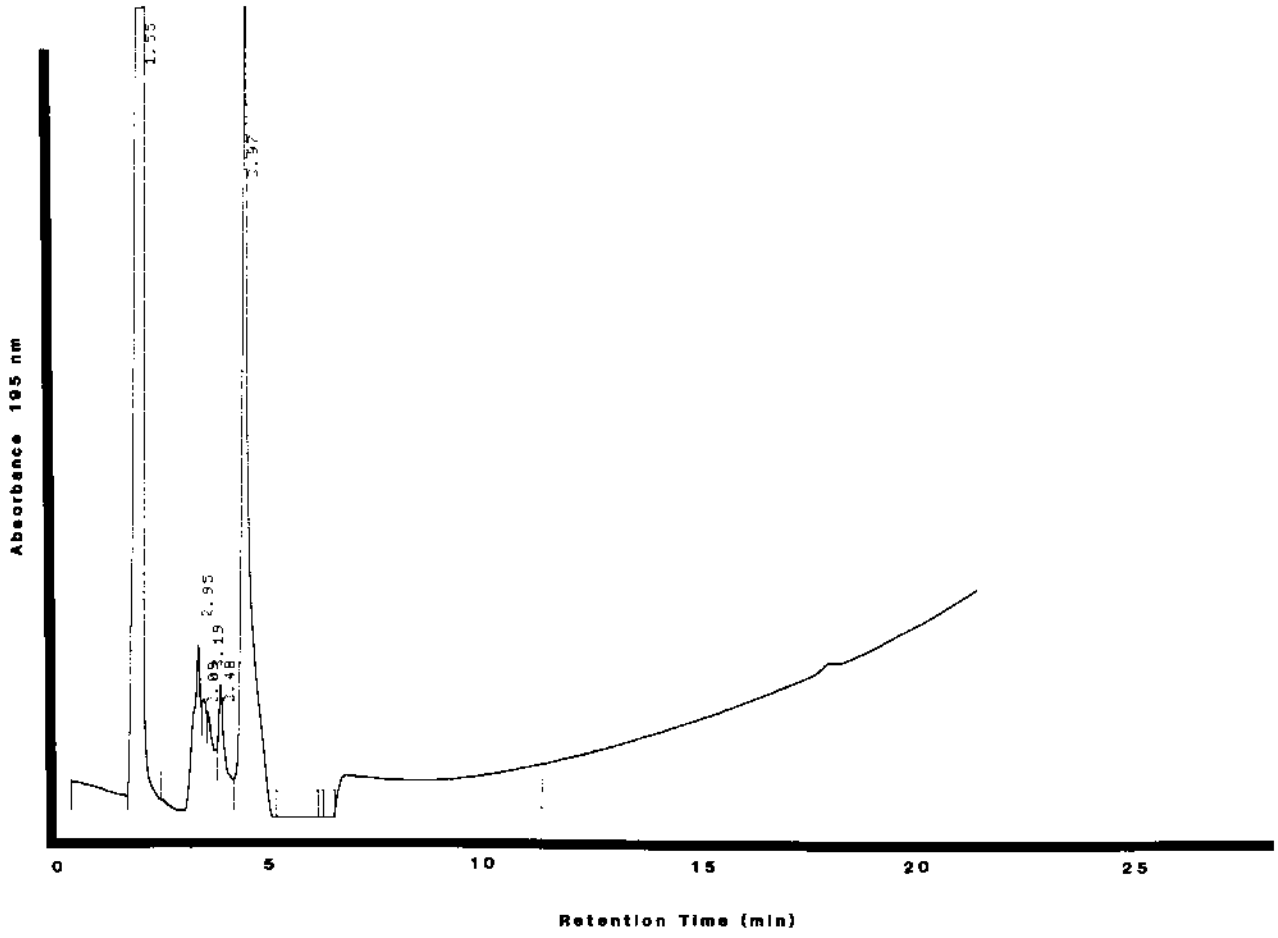

Absorbance 195 nm
1.55
2.95
3.19
3.48
3.97
0
5
10
15
20
25
Retention Time (min)

TABLE V

HUMAN SERUM SAMPLES ANALYZED BY GAS-LIQUID CHROMATOGRAPHY (GLC)

Fungal Disease	No. of Samples
Aspergillosis	6
Blastomycosis	5
Candidiasis	7
Coccidioidomycosis	37
Histoplasmosis	7
Controls	25

fungal disease sera were obtained from individual patients with documented fungal disease. The chromatograms shown in the following pages are representative patterns that were obtained by running each patient's sample in triplicate.

Figure 29 shows the gas-liquid chromatogram of the peracetylated aldonitrile acetate (PAAN) derivatives of the carbohydrates contained in a control serum sample. The peak at a retention time of 16.43 minutes is glucose; the peaks with retention time at 10.68 (d-lyxose) and 11.00 minutes (d-ribose) respectively, were internal standards, and the rest of the chromatographic peaks are as yet unidentified. Figure 30 shows the gas-liquid chromatogram of the PAAN derivatives contained in a serum sample from a patient with aspergillosis. The chromatogram looks similar to the one obtained from the normal serum sample. However, there is a disappearance of the glucose peak and the appearance of a peak with a retention time of 16.11 minutes which corresponds to mannose. Therefore, even though the GLC pattern of the aspergillosis serum is similar to the control GLC pattern, it is still different. As shown in Figure 31, the gas-liquid chromatogram obtained from the derivatization of a serum sample from a patient with blastomycosis is quite complex in comparison to that obtained from normal serum. The major differences from that of normal serum is the presence of a peak at 16.06 minutes, which is mannose, and the absence of a glucose peak. The rest of the chromatogram that occurs

Fig. 29--Gas-liquid chromatograms (hydrogen-flame detector) of the PAAN derivatives of the carbohydrates contained in a control serum sample using OV-17 on Chromsorb W (2 mm i.d. x 1.23 m), nitrogen flow of 22 ml/min, and programmed at 130° to 300° at 5°/min. Injector and detector set at 280°.

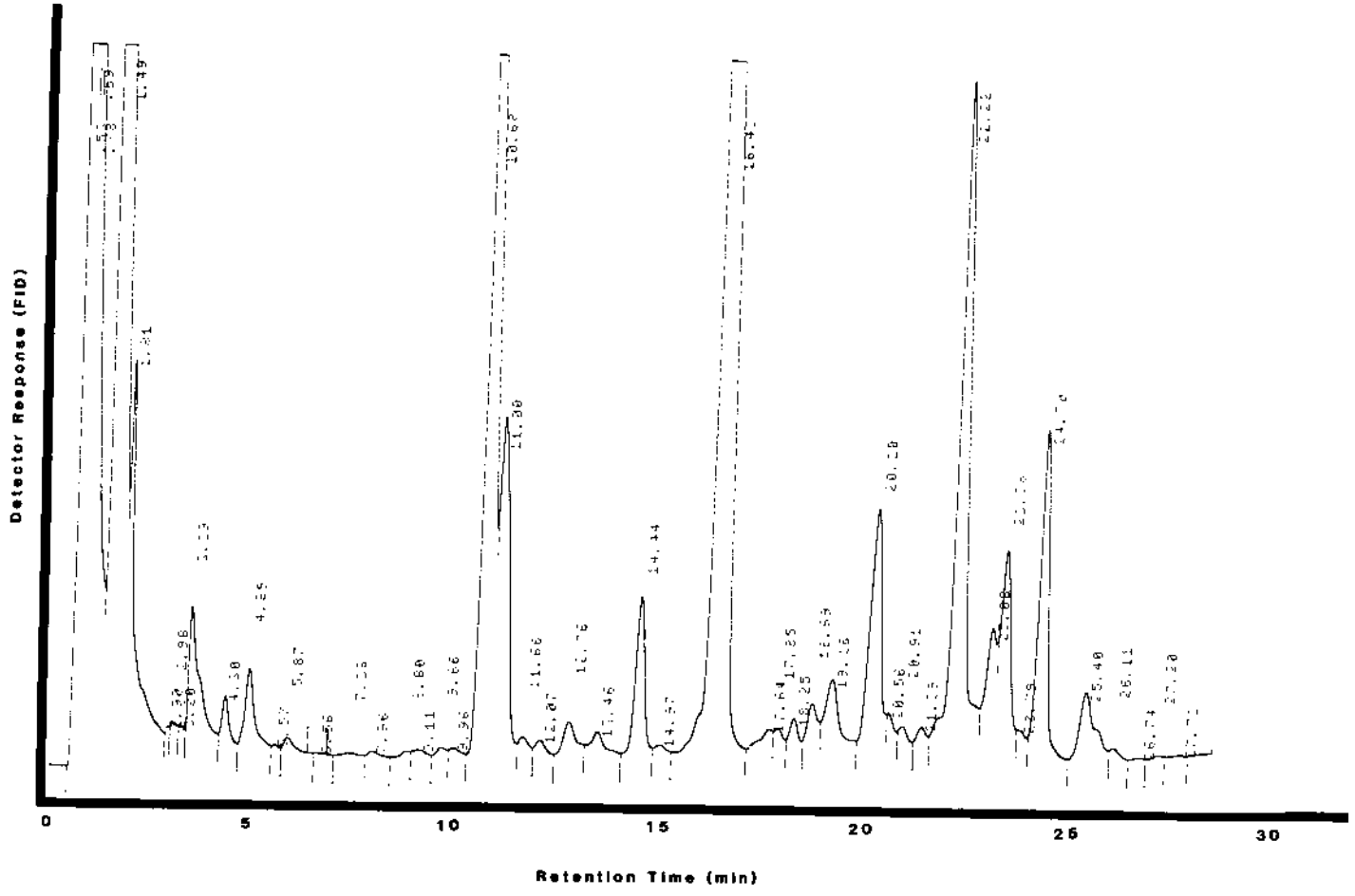

Detector Response (FID)
0
5
10
15
20
25
30
Retention Time (min)

Fig. 30--Gas-liquid chromatograms (hydrogen-flame detector) of the PAAN derivatives of the carbohydrates contained in a serum sample from a patient with aspergillosis using OV-17 on Chromsorb W (2 mm i.d. x 1.23 m), nitrogen flow of 22 ml/min, and programmed at 130^{o} to 300^{o} at 5^{o}/min. Injector and detector set at 280^{o}.

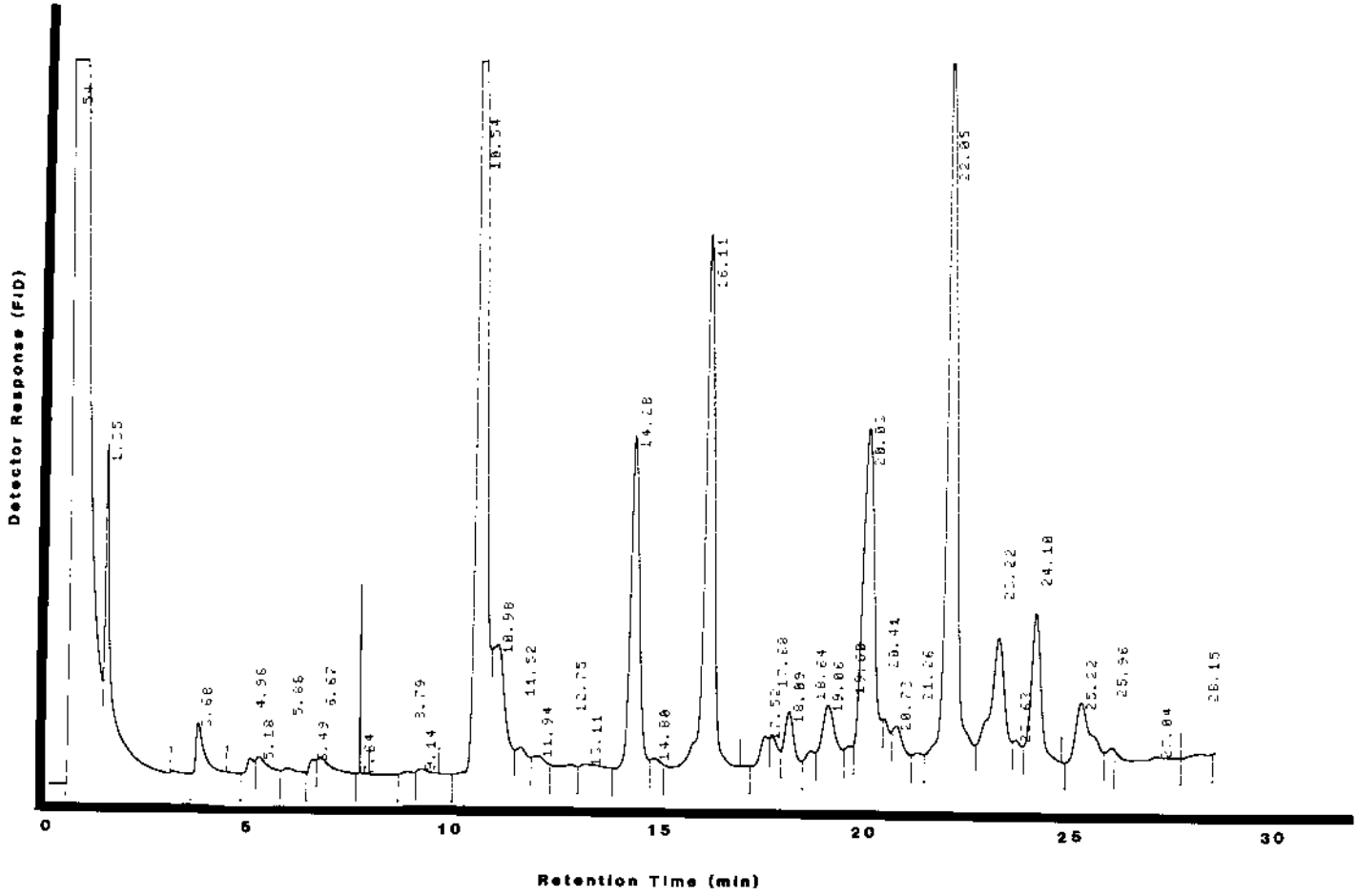
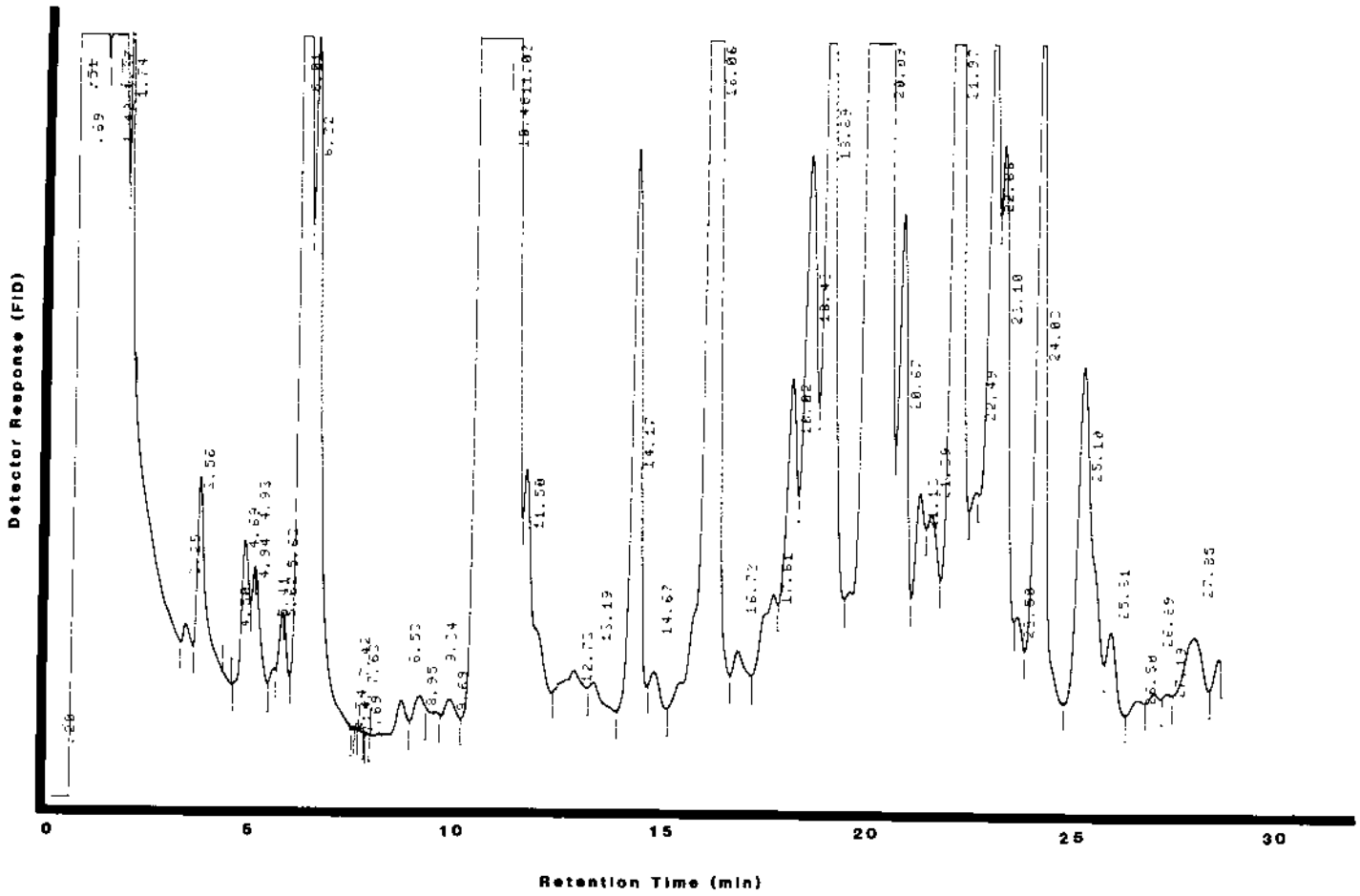

Detector Response (FID)
0
5
10
15
20
25
30
Retention Time (min)

Fig. 31--Gas-liquid chromatograms (hydrogen-flame detector) of the PAAN derivatives of the carbohydrates contained in a serum sample from a patient with blastomycosis using OV-17 on Chromsorb W (2 mm i.d. x 1.23 m), nitrogen flow of 22 ml/min, and programmed at 130^{o} to 300^{o} at 5^{o}/min. Injector and detector set at 280^{o}.


Detector Response (FID)
0
5
10
15
20
25
30
Retention Time (min)

after a retention time of approximately 17 minutes is very complex and drastically different from the pattern that was obtained with the normal serum sample. As with the sample obtained from the patient with blastomycosis, Figure 32 shows a derivatized sample that was obtained from a patient with candidiasis. Again, there is the appearance of a large mannose peak at 16.16 minutes and the lack of a glucose peak. Also, as with Figure 31, Figure 32 shows a fairly complex chromatogram after a retention time of 17 minutes, and, at first glance, it is hard to tell whether it is less or more complex than the same area on the chromatogram from the patient with blastomycosis. Following the already established trend, Figure 33 demonstrates the gas-liquid chromatographic pattern obtained from the derivatization of a sample from a patient with histoplasmosis. Again, there is the appearance of a large mannose peak at 16.15 minutes and a fairly complex chromatogram after retention time of 17 minutes. As with the prior chromatograms (Figures 30, 31, 32), the area of complexity occurring between retention times of 17 and 30 minutes contains peaks which, as of yet, have not been identified. Figure 34 shows the gas-liquid chromatogram obtained from the derivatization of a serum sample from a patient with coccidioidomycosis and several important peaks are demonstrated here--first, the peak at 15.95 minutes which corresponds to mannose, and the peak at 16.36 minutes which correlates with glucose. The peak of primary interest

Fig. 32--Gas-liquid chromatograms (hydrogen-flame detector) of the PAAN derivatives of the carbohydrates contained in a serum sample from a patient with candidiasis using OV-17 on Chromsorb W (2 mm i.d. x 1.23 m), nitrogen flow of 22 ml/min, and programmed at 130° at 5°/min. Injector and detector set at 280°.

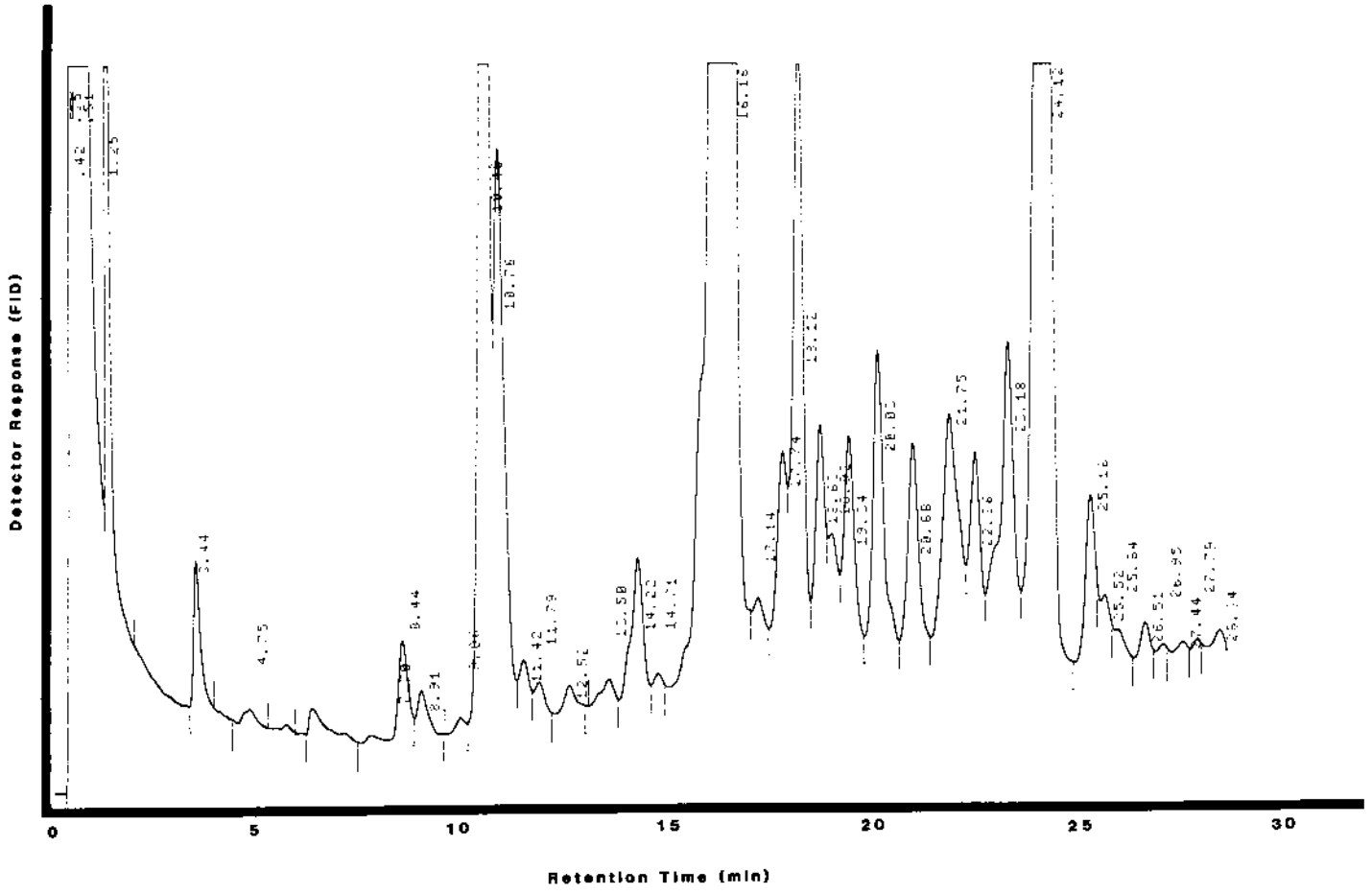
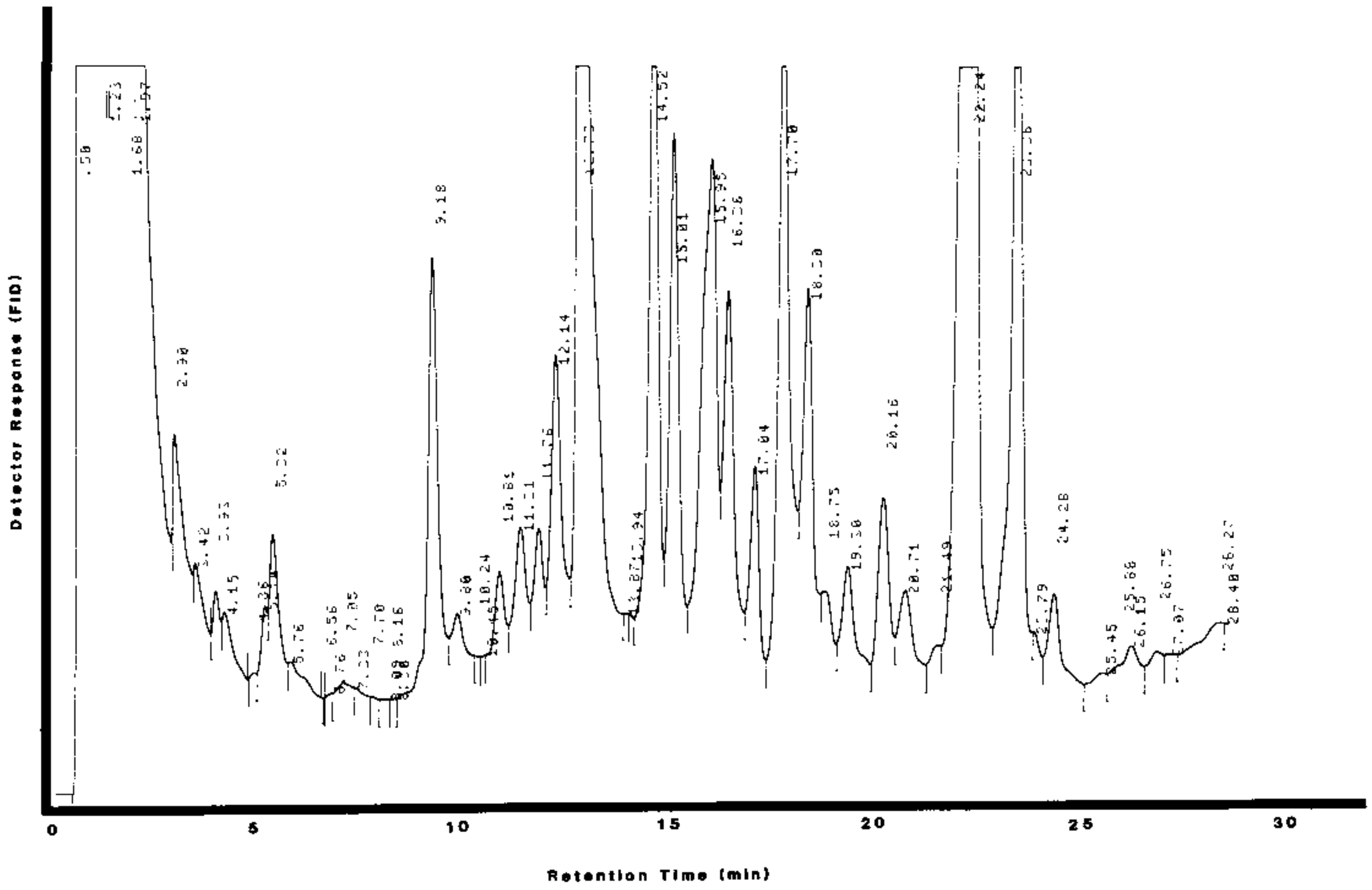

Detector Response (FID)
0
5
10
15
20
25
30
Retention Time (min)

Fig. 33--Gas-liquid chromatograms (hydrogen-flame detector) of the PAAN derivatives of the carbohydrates contained in a serum sample from a patient with histoplasmosis using OV-17 on Chromsorb W (2 mm i.d. x 1.23 m), nitrogen flow of 22 ml/min, and programmed at 130^{o} to 300^{o} at 5^{o}/min. Injector and detector set at 280^{o}.

Detector Response (FID)
Retention Time (min)
0
5
10
15
20
25
30
.54
1.86
2.58
3.58
4.66
5.64
5.75
6.25
9.43
9.67
11.51
12.64
13.27
14.25
14.61
16.15
17.07
17.63
18.45
18.94
19.41
20.13
20.69
21.55
22.01
22.45
24.05
25.16
26.55
27.02
27.96

Fig. 34--Gas-liquid chromatograms (hydrogen-flame detector) of the PAAN derivatives of the carbohydrates contained in a serum sample from a patient with coccidioidomycosis using OV-17 on Chromsorb W (2 mm i.d. x 1.23 m), nitrogen flow of 22 ml/min, and programmed at 130^{o} to 300^{o} at 5^{o}/min. Injector and detector set at 280^{o}.


Detector Response (FID)
Retention Time (min)
0
5
10
15
20
25
30
.58
1.68
2.98
3.42
4.15
5.32
5.76
6.56
7.05
7.70
9.18
10.24
10.84
11.11
12.14
14.52
15.81
16.38
17.04
18.30
18.75
19.38
20.16
20.71
21.49
22.24
24.28
25.45
25.88
26.15
26.75
28.48

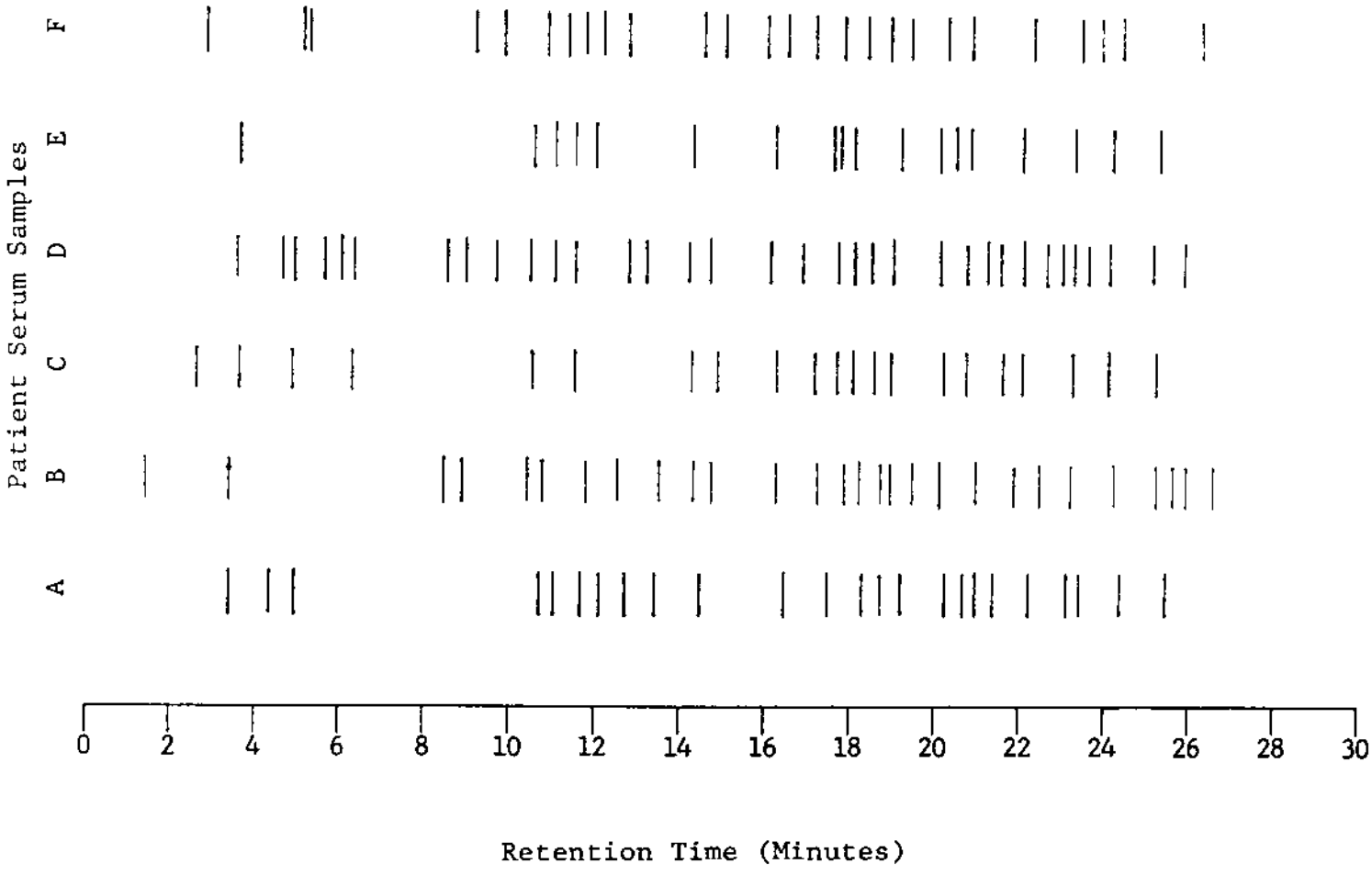
is the one that occurs at 15.01 minutes and has been identified as 3-0-methyl mannose, a sugar monomer that is, among the fungi, unique to the cell wall of Coccidioides immitis.

For purposes of comparison, Figure 35 shows a schematic representation of the peaks that were obtained from the derivatization of the deproteinized serum sample from normal as well as those from the sera of individuals with various fungal diseases. It is first noteworthy that the derivatization of a sera results in quite complex chromatograms. From 0 to 10 minutes of retention time, all of the sera showed relatively few peaks. Only in the blastomycotic sera (D) were there what appeared to be a large group of possibly significant early peaks. These peaks have not yet been identified.

From retention times of 8-10 minutes, only sera from the patients with candidiasis (B), blastomycosis (D), and coccidioidomycosis (F) had any peaks. There appears to be some overlap between these disease states in this region, and it appears that when these peaks are identified they would not be useful for the diagnosis of the individual disease states, but might be significant for alerting the investigator to the possible presence of a fungal disease.

In the region of the chromatogram bounded by retention times of 11-14 minutes, histoplasmosis (C) and aspergillosis (E) showed the fewest number of peaks. As with the area

Fig. 35--Schematic representation of the gas-liquid chromatograms of the PAAN derivatives of the carbohydrate components of serum from patients with various fungal diseases: A, normals; B, Candidiasis sera; C, Histoplasmosis sera; D, Blastomycosis sera; E, Aspergillosis sera; and F, Coccidioidomycosis sera. The vertical bars represent the average retention times of carbohydrate peaks from triplicate runs (±0.15 min) for each fungal disease.


Patient Serum Samples
F
E
D
C
B
A
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Retention Time (Minutes)

from 8-10 minutes of retention time, there was some overlap of peaks and hence a lack of discriminatory ability with the peaks found in this region. In the area from 14 minutes of retention time on out to the end of the chromatograms, all of the chromatograms are quite complex in nature. It appears, however, that the patterns are distinct and hence could be used to recognize normal sera versus the various disease states. Also, as stated in the discussion of Figure 34, the presence at 15 minutes of a peak in the gas-liquid chromatographic pattern of coccidioidomycosis, which corresponds to 3-0-methyl mannose, would be a useful way to discriminate coccidioidomycosis serum from the sera of other disease states and/or a normal serum sample.

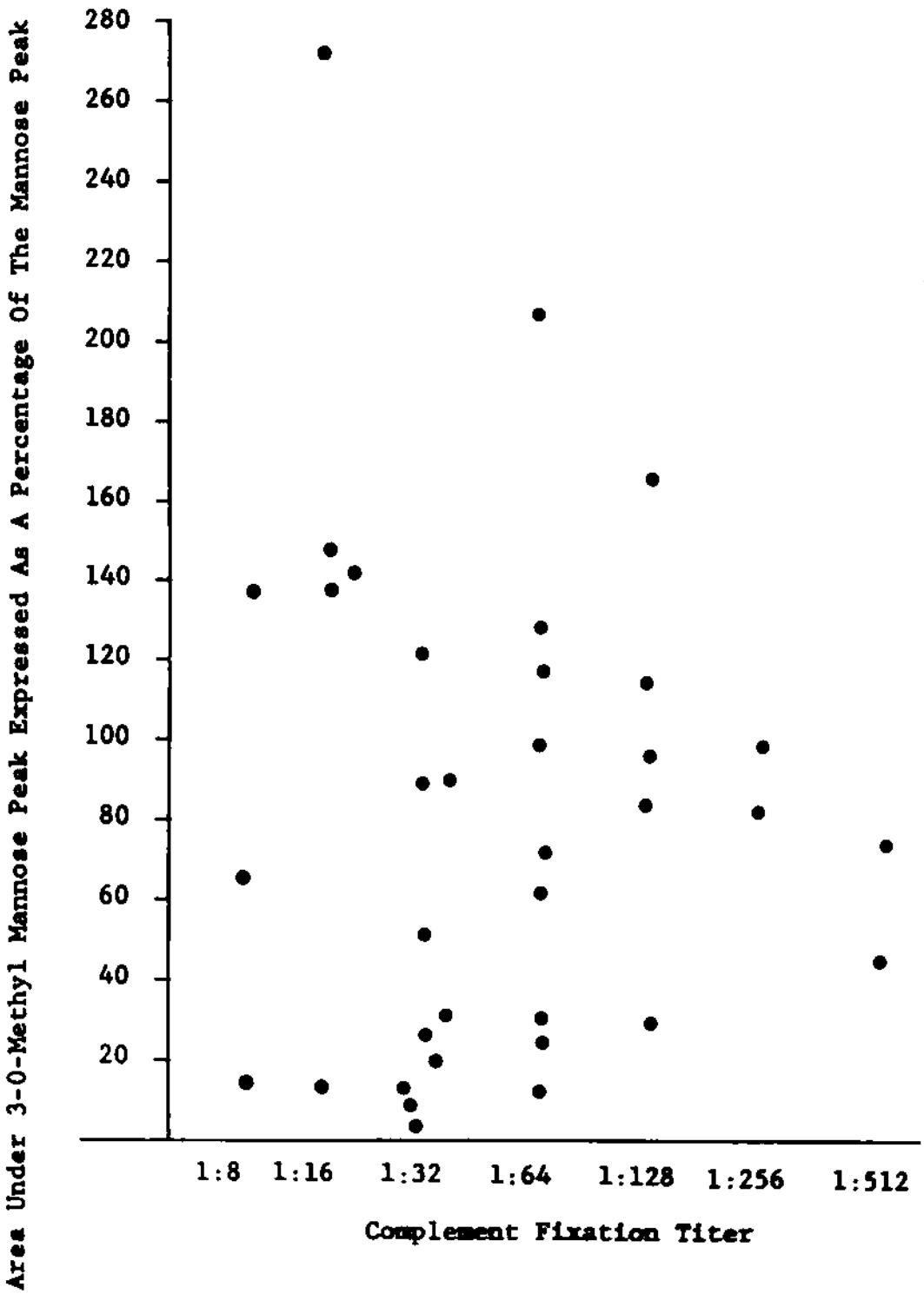
Figure 36 shows the ratio of the area under the mannose peak as expressed a percentage of the total area of all the peaks for the fungal disease states tested. Although the number of points are small, it appears that possibly in looking at this ratio one would be able to get an idea as to the infecting fungal agent. If the serum demonstrated a low mannose to total peak ratio, it would most likely be a chance of either having coccidioidomycosis or blastomycosis. These two could then be differentiated by the presence or absence of 3-0-methyl mannose, which would indicate either coccidioidomycosis and/or blastomycosis. A relatively high ratio of mannose to total number of peaks would indicate candidiasis. Whereas, both histoplasmosis and aspergillosis

Fig. 36--A comparison of the values obtained by determining the area under the mannose peak expressed as a percentage of the total areas of all the peaks in the chromatogram for each fungal disease sera group.

Coccidioidomycosis Blastomycosis Histoplasmosis Aspergillosis Candidiasis
5
10
15
20
25
30
35
Area under mannose peak expressed as a percentage
of the total area of all peaks.

sort of fit in between the high end of candidiasis and the low end of coccidioidomycosis and blastomycosis. Finally, Figure 37 shows the plot of the area under the 3-O-methyl mannose peak expressed as the percentage of the mannose peak versus the complement fixation titer of the coccidioidomycosis sera that were run in the GLC analysis. It is obvious that there is no correlation between the amount of 3-O-methyl mannose and/or mannose and the complement fixation titer of the sera. Therefore, in order to diagnose the presence of Coccidioides immitis in a patient through the derivatization of serum, the key would be the presence or absence of the 3-O-methyl mannose peak.

Fig. 37--Differential results from plotting the area under the 3-O-methyl mannose peak expressed as a percentage of the mannose peak against the complement fixation titer for the coccidioidomycosis patient sera.


Area Under 3-O-Methyl Mannose Peak Expressed As A Percentage Of The Mannose Peak
280
260
240
220
200
180
160
140
120
100
80
60
40
20
1:8
1:16
1:32
1:64
1:128
1:256
1:512
Complement Fixation Titer

CHAPTER V

DISCUSSION

High Performance Liquid Chromatographic Analysis of Fungal Antigens

A variety of coccidioidin preparations have been reported in the last three decades. As developed by C. E. Smith and co-workers in the 1940's, coccidioidin is comprised of filtrates of cultures of mycelial growth of Coccidioides immitis and contains heat labile complement fixing (CF) as well as heat stable precipitin and delayed hypersensitin skin test activities (2, 117, 119, 120). More recently, culture filtrates and toluene lysates of mycelia (56, 57, 96, 97, 98), spherules (75, 76, 77), and a variety of extracts and fractions (7, 73, 132, 135, 137, 139) have also been shown to be reactive in infected or sensitized individuals and animals. Whether the same biologically active components are present in these various preparations is presently unknown. Moreover, little attention has been given to the isolation and characterization of coccidioidin activities. Therefore, the first portion of this study was undertaken to determine if the use of high performance liquid chromatography would be suitable for the separation of the components of the various fungal antigen preparations.

From the data presented in the results section of this study, it is evident that the use of size exclusion chromatography will not be very useful in the separation of complex fungal antigen preparations. In looking at the chromatograms obtained from the size exclusion chromatographic separations of histoplasmin H-42, histoplasmin HKC-43, blastomycin KCB-26, and coccidioidin (toluene lysate), it can be seen that baseline separation did not occur for any of the component peaks, and further, the peak shapes indicate that the peaks were heterodisperse rather than homogeneous in nature. Only in the case of simpler antigenic preparations, such as b-ASWS and c-ASWS, would the technique appear to have at least minimal benefit in a primary separation. For the b-ASWS (Figure 4), it can be seen that the baseline separation or near baseline separation was achievable on two peaks, and as such, separation of these two peaks would be possible with the technique of size exclusion chromatography. The separation achieved with c-ASWS (Figure 6), although demonstrating fewer peaks than were seen with the b-ASWS, still achieved baseline separation for one peak. However, even though the technique was not readily useful in the separation of complex fungal antigen preparations, a comparison via a schematic representation (Figure 7) indicates that it is possible to utilize the technique (SEC) to obtain at least an idea of whether or not these varied fungal antigen preparations have component(s) in common with one another.

This could explain the cross-reactivity that has been seen in serologic testing with these various preparations.

The chromatographic patterns generated through the use of ion-exchange chromatography of the various fungal antigens showed that this technique gave better resolution for a primary separation of the various components of the fungal antigens. For the four complex antigenic preparations (histoplasmin H-42, histoplasmin HKC-43, blastomycin KCB-26, and coccidioidin (toluene lysate)), the ion-exchange chromatographic patterns showed that baseline separation was obtained for at least two major component peaks in each of these complex antigenic mixtures. It is probable that the baseline separation for these two major components in each case was due to a combination of both a size exclusion and a minimal of electrostatic interactions taking place even though the two major components were retained by the ion-exchange matrix. With further study, it may be possible to increase the resolution in the ion-exchange portion of the separation of the fungal antigen preparations by using ion-exchanges with larger pore-diameters. Thus, overcoming possible pore-diameter limitations which cause a stagnation of mobile phase mass transfer phenomena. In the case of the more simple fungal antigen preparations (b-ASWS, c-ASWS), the ion-exchange chromatograph patterns revealed the same base-line separation of two major components, as was seen with the

more complex antigenic preparations, but also fairly good separation of those components that were in fact bound to the matrix of the ion-exchange material. Thus, it looks as if ion-exchange chromatography will be an excellent way of approaching an initial separation of the components of both complex antigenic mixtures as well as simpler antigenic preparations. Additionally, since ion-exchange makes use of non-denaturing, aqueous phases for both the mobile and the elution phase, it should be possible to do the primary separation using this methodology. Once collected, the individual components could be tested to determine their biologic activity and structural make-up.

The most interesting chromatographic patterns were generated with the reverse-phase column used in the study. All of the complex fungal antigen preparations that were subjected to reverse-phase chromatography showed a large component peak that eluted before the initiation of the gradient portion of the chromatographic separation. In the case of coccidioidin, and in addition to the major peak, there was also a second smaller component peak that eluted before the beginning of the gradient section of the chromatogram. In all cases, the complex antigen mixtures were shown to be composed of multiple components all of which separated only after the beginning of the gradient elution phase of the separation. Thus, in comparison with the other two methods of high performance liquid chromatographic separation

employed in the study, the reverse-phase separation demonstrated the best resolution of multiple components within these complex fungal antigen preparations. In looking at the chromatographic patterns obtained with b-ASWS and c-ASWS, the reverse-phase patterns demonstrated that these antigen preparations are less complex than the culture filtrate and toluene lysate preparations. Reverse-phase chromatography will be useful in two areas of study of fungal antigen preparations: 1) fingerprinting of various antigenic preparations for the purpose of standardization, and 2) separation and collection of individual components for structural and immunological analysis. As was proposed for the two-dimensional immunoelectrophoresis technique (62, 63), the resolution of reverse-phase chromatography can be directed toward standardizing antigen preparations and separating and collecting species-specific antigens. Once collected, the antigens can be subjected to structural analysis. However, since reverse-phase chromatography uses organic solvents as eluants, it is not known whether or not the interaction of the various components in the antigenic preparations with the organic solvents will damage those components structurally. Further research is necessary to determine if the resolving power of reverse-phase chromatography will allow separation of immunologically active components from these complex fungal antigen preparations.

Another area of high-pressure liquid chromatographic separation which was introduced in this particular study was in the use of a primary bonded-amine column. This particular type of column was used to obtain the data presented only with the c-ASWS antigen preparation. The chromatographic pattern obtained with the use of this column for the separation of c-ASWS showed that it produced very sharp, well separated peaks. Until this study, normal phase chromatography using a bonded primary amine column has been restricted to the separation of carbohydrate monomers. However, this study indicates a usefulness in the separation of mannoproteins and other large carbohydrate-containing components. This potential use of this column in the study of fungal antigen preparations will be pursued further by future research work.

From the data presented in this study, it can be concluded that future work on the separation fungal antigen preparations should include work on a system for coupled multiple-column separation of fungal antigens. From the standpoint of solvent compatibility, the combination of an anion-exchange column coupled to a reverse-phase column would be a very functional binary column combination. The combination of size exclusion and electrostatic partitioning contributed by the anion-exchange column plus the hydrophobic interaction by the reverse-phase column would lead to good resolution of complex fungal antigen preparations. Again,

separation and collection of individual components for structural analysis could lead to the development of species-specific antigenic preparations for use as diagnostic reagents. However, it is not yet known if this binary column combination will adversely affect the immunological activity of isolated components. Only future research will be able to provide an answer to this very important question.

Preliminary Structural Characterization of c-ASWS

Infra-red spectral analysis of c-ASWS indicated that this particular preparation definitely contained both protein and carbohydrate (mannans). Otherwise, the IR spectra did not provide much information concerning the nature of the molecule, especially the nature of the linkages involved. Amino acid analysis and subsequent beta-elimination reaction analysis indicated that there might be the presence of glycoproteins or mannoproteins because the results of these two studies show that all of the available threonine and half of the available serine residues were involved in oligoglycosidic linkages with carbohydrates. However, at this point, it still remains to be seen whether this is a true glycoprotein, in that it has a protein core to which are attached carbohydrate units of varying links, or if this is indeed made up of polysaccharide cores to which are attached small protein subunits. Not unlike other fungal antigen preparations, this antigen has been shown to be predominantly carbohydrate in nature with about half as much peptide and/or protein being present. It has a small amount of lipid associated with it, and the carbohydrate portion is made up of monomers of mannose, galactose, and 3-O-methyl mannose. From the fact that it reacts with con A, it can be stated that the antigen preparation contains components with single, terminal, non-reducing glycosyl residues. These residues

are attached to the main carbohydrate backbone by α-D-(1→6)-glycosidic linkages, as con A preferentially interacts with this type of linkage as opposed to glycosidic linkages which involve secondary hydroxyl groups.

The chromatography on the 4% agarose under reducing conditions showed that the antigen preparation was made up of at least four distinct components containing both protein and carbohydrate. Two of these components fell outside the linear range of that particular agarose and thus were concluded to have a molecular weight of greater than 200,000. However, the other two fell within the linear range of the gel and demonstrated calculated molecular weights of slightly more than 59,000 and 32,000. Although the chromatography under reducing conditions demonstrated only four components, the N-terminal analysis data suggested that there might be at least eight protein N-terminals present within the molecule. This can be interpreted to mean that rather than being glycoproteins with a protein core, the components are actually large polysaccharide units which have individual protein chains attached through an oligoglycosidic link with either serine or threonine residues.

As already discussed in the preceding section, the data clearly indicated that either ion-exchange chromatography or reverse-phase chromatography would be the best method for the separation and subsequent analysis of the components of this

particular antigen. Although the size exclusion chromatographic patterns did show a separation of at least two components, both ion-exchange and reverse-phase chromatographic patterns showed a separation of a minimum of four and a maximum of eight component peaks being contained in the c-ASWS preparation. Therefore, with c-ASWS, further separation research should involve the use of a reverse-phase column with an isocratic buffer containing guanidine HCL. In this way, the guanidine HCL could be used to provide individual components for better partitioning in the stationary-phase of the reverse-phase column. This should allow the high resolution of the reverse-phase column to be coupled with a buffer system that should not damage structurally the various antigenic components so that after separation, they would still retain their immunologic activity.

Unfortunately, this study generated a small amount of data to indicate that the use of a bonded primary amine column would be useful in the separation and subsequent collection of the components of this antigen. Since c-ASWS has been shown to contain a number of carbohydrate-containing components, future work should involve looking at the possible uses of the bonded primary amine column in the separation and characterization of fungal antigen preparations.

Therefore, on preliminary analysis, it appears that the c-ASWS antigen preparation is composed of a minimum of four components that contain both carbohydrate and protein. It is not yet known whether or not each of these components has the carbohydrate and protein linked together in 0-glycosidic linkages. This study has shown that c-ASWS contains mannose, 3-0-methyl mannose and galactose. Thus, the results are quite compatible with other analyses of coccidioidin skin test fractions which have been reported to be comprised of predominantly mannopeptides (or protein) containing smaller amounts of 3-0-methyl mannose and other sugars including glucose and galactose (7, 97, 102). The peptide or protein component(s) have been reported to be slightly enriched in proline (7) and to contain, in addition, a variety of amino acids (7, 97). This study is in agreement with the presence of a variety of amino acids, but there was no proline, cysteine, or tryptophan evident in the amino acid analysis of c-ASWS. The coccidioidin skin test reactive fraction has been assigned a molecular weight of 31,700 in one study (97), but shown to be in the range of 10,000 to 60,000 in another study (7). C-ASWS has been shown in this study to contain a component with an assigned molecular weight of 32,000 and a second component of molecular weight of 59,485. The component with the molecular weight of 31,000 may be similar to the polysaccharide reported in other studies (7, 97). The study of Anderson et al. (7) showed further that material

with a molecular weight of greater than 100,000 was not adequate for the skin test. Therefore, it may be that the other two components of c-ASWS, whose molecular weights have been shown to be greater than 200,000, may not contribute to the skin test reactivity of c-ASWS.

Until methylation analysis is done on c-ASWS, the nature of the carbohydrate linkages will be unknown. However, it is probable that c-ASWS is composed of a backbone of α-(1→6)-linked D-mannopyranose residues with galactose linked through either the C-2, C-3, or C-6 of the D-mannose residues. The 3-O-methyl mannose is most likely in a position as a terminal, non-reducing, end group that is attached to the backbone via an α-(1→2)-linkage. The protein is probably attached to this highly branched heteromannan through O-glycosidic linkages to threonine and serine residues.

Rapid Diagnosis of Fungal Infection by Gas-Liquid Chromatography: Analysis of Sugars in Normal and Infected Serum Samples

Amundson et al. (6) presented data on the use of gas-liquid chromatographic (GLC) analysis of sugars in cerebrospinal fluid for the diagnosis of meningeal cryptococcosis and thalamic astrocytoma. The authors were able to obtain a reproducible chromatogram from a normal human cerebrospinal fluid which they compared to chromatograms generated from infective CSFs (cryptococcosis and thalamic astrocytoma). The comparison showed a decrease in peak size from the normal chromatogram, as well as the appearance of a new peak in the chromatograms from the infected CSF. However, both the peak size depression and the appearance of a new peak occurred in the CSF from patients with meningeal cryptococcosis as well as those from patients with thalamic astrocytoma.

Schlossberg et al. (111) used GLC to analyze CSF from patients with cryptococcal and viral meningitis. Similar EC-GLC patterns were obtained from all patient specimens with cryptococcal meningitis. This pattern was different from the patterns obtained from EC-GLC analysis of CSF from both normal controls and viral meningitis patients. Even though the authors failed to identify the compounds responsible for the EC-GLC patterns, that pattern or imprint could still be used in the diagnosis of cryptococcal meningitis. This work was expanded by the study of Craven et al. (28) who looked at

CSF patients with tubercular, cryptococcal, and viral meningitis. They found that two distinct EC-GLC patterns were obtained from analysis of the CSF from patients with cryptococcal meningitis. Each pattern was distinct from the patterns produced from the analysis of tubercular and viral CSF. Additionally, the effects of treatment with amphotericin B on the EC-GLC pattern showed that the technique could be used to follow and document the success of a course of antifungal therapy.

Miller et al. (86) used GLC to analyze sera from patients with candidiasis. The patterns generated from the analysis of sera taken from patients with candidiasis were significantly different from the pattern generated from analysis of sera from normal patients. Again, components producing the peaks were not identified, but the possibility of using GLC in the diagnosis of candidiasis was reported. Monson and Wilkerson (90, 91) showed that candidiasis could possibly be diagnosed by the GLC analysis of patient sera looking for the presence of mannose, a sugar not usually found in human sera, but an integral part of the cell wall of Candida albicans. This idea was exploited by other workers (40, 48, 72, 106, 137, 138) who looked for the presence of the fungal metabolite, arabinitol, in human sera to diagnose the presence of candidiasis. By using arabinitol as a marker for the presence of Candida albicans, Gold et al. (48) were able to diagnose candidiasis in patients who otherwise had

negative blood culture results.

Although this study focused on the GLC analysis of sera from patients with coccidioidomycosis, it also included small numbers of sera from patients with aspergillosis, blastomycosis, candidiasis, and histoplasmosis. Except for the GLC pattern obtained from the aspergillosis sera, the patterns obtained from the other fungal disease sera were quite complex and yet different from each other and from the pattern obtained from normal control sera. Since the patterns obtained were both complex and different from each other, it is possible that the diagnosis of various fungal diseases could be made by computer analysis of the fingerprints obtained from the GLC analysis of deproteinized sera. A computer could be used to compare the chromatograms obtained from the GLC analysis of individual patient serum with the chromatograms of the GLC analysis of various fungal disease sera already analyzed. In this way, the computer could match the patient's serum chromatogram with a specific fungal disease state, and thus provide a non-invasive diagnostic technique for fungal diseases. Additionally, the GLC could be coupled to a mass spectrometer (74) to determine the structure of peaks unique to a specific fungal disease so that the accuracy of the computer analysis would be enhanced.

As with the work of Monson and Wilkerson (90, 91), the results of this study showed the presence of mannose in the sera taken from patients with various fungal diseases. The

data showed that the ratio of the mannose peak area/total peak area was largest for the sera taken from patients with candidiasis and lowest from patients with both coccidioidomycosis and blastomycosis. This ratio was intermediate for patients with either aspergillosis or histoplasmosis. This correlates fairly well with the results obtained by Monson and Wilkerson (91) in their study of sera from patients with fungal disease. Thus, it appears that a general diagnosis of a fungal infection could be made if the GLC analysis of patient's serum demonstrated the presence of mannose. Further, a high ratio of mannose peak area/total peak area would indicate that the patient had candidiasis. A lower ratio would indicate either the presence of coccidioidomycosis or blastomycosis while an intermediate level could indicate either aspergillosis or histoplasmosis.

Taking the analysis one step further, the study was further able to demonstrate the presence of a methylated sugar, 3-O-methyl mannose, in the GLC patterns from the patients with coccidioidomycosis. Among the fungi, this sugar has only been reported to be in the cell wall of Coccidioides immitis. However, the sugar has been reported in the lipopolysaccharides of Mycobacterium phlei (49) and of Klebsiella and Escherichia coli (93). Therefore, it is possible that the presence of 3-O-methyl mannose might be due to a gram negative bacteremia. However, this should not cause a problem in the use of 3-O-methyl mannose as a marker

for coccidioidomycosis for the following two reasons: 1) this methylated sugar was only 2% of the lipopolysaccharide and resided in that portion of the LPS that did not react antigenically, and 2) a diagnosis of coccidioidomycosis would involve both 3-0-methyl mannose and mannose. The mannose would not be present in patient's serum with a gram negative bacteremia. Thus, the presence of the methylated sugar, 3-0-methyl mannose, along with the presence of mannose provides a marker for the diagnosis of coccidioidomycosis by GLC analysis of sera.

In summary, this study shows that the analysis of deproteinized patient serum by GLC will result in complex fingerprints being obtained from the various fungal disease states. The GLC patterns obtained will be significantly different from each other, thus, establishing the possibility that computer assisted pattern analysis could be used not only to diagnose the fungal disease but also, to determine the identity of the organism causing the disease. Further, all of the fungus diseases studied showed the presence of mannose in patient serum. With a larger data base, it should be possible to look at the ratio of mannose peak area/total peak area and determine the etiological agent causing the disease in the patient. Finally, in the case of patients with coccidioidomycosis, a unique carbohydrate monomer, 3-0-methyl mannose, was demonstrated in the GLC patterns taken from the patient sera. Further work is needed to establish

whether or not the presence of this monomer is consistent in all coccidioidomycosis patients, whether the relative levels of the monomer can be correlated with the various stages of the disease, and whether or not the levels of this particular carbohydrate could be of prognostic value to the physician.

APPENDIX

In the calculations used to determine the molecular weight of the four component peaks of c-ASWS, the following value was determined:

$$F(v) = \frac{Ve^{1/3} - Vo^{1/3}}{Ve^{1/3} - Vo^{1/3}} = \frac{(Ve/Vt)^{1/3} - (Vo/Vt)^{1/3}}{1-(Vo/Vt)^{1/3}}$$

where Ve = the elution volume of the sample substance

Vo = void volume as determined with Blue Dextran

Vt = total volume as determined with DNP-ALA

This value F (v) is related to the cube root of the molecular weight, $M^{1/3}$, by the following: $F(v) = b - aM^{1/3}$ where b = intercept and a = slope of a line determined by plotting the values of F (v) against the values of $M^{1/3}$ for a set of molecular weight standards. The intercept, slope, and correlation coefficient values were determined by performing a linear regression analysis of the values F (v) = x vs. $M^{1/3}$ = y for the molecular weight standards. These values for the molecular weight standards are shown in Table VI.

Regression analysis was run on the values F (v) = x and $M^{1/3}$ = y for the protein molecular weight standards and for the Dextran molecular weight standards. This analysis yielded the values for the slope, intercept, and correlation coefficient for the two lines generated by the two sets of data. This interaction is related to the values F (v) and $M^{1/3}$ by the following equation: $F(v) = b - aM^{1/3}$ where b = intercept and a = slope of the line. For the protein molecular

weight standards, this equation was as follows:

(A) $F(v) = 0.7219464 - 0.0074835\ M^{1/3}$. For the Dextran molecular weight standards, this equation was as follows:

(B) $F(v) = 0.796775 - 0.0101086\ M^{1/3}$. Therefore, in order to calculate a molecular weight for a sample component, the two equations would be rewritten as follows:

$$M^{1/3} = \frac{0.7219464 - F(v)}{.0074835}$$ and

$$M^{1/3} = \frac{0.79141 - F(v)}{.0101086}$$

For the c-ASWS, the first two components co-chromatographed with the Blue Dextran and were, thus, outside of the linear range of the column (10,000 - 200,000). The next two components had F (v) of 0.3968056 and 0.4704861, respectively. When these values were put into the two formulas that relate molecular weight to F (v), the following molecular weight values were determined:

Using equation A: 82,016 and 37,940

Using equation B: 59,485 and 31,999

Since the components were determined to contain carbohydrate in addition to protein, it was felt that the values of 59,485 and 31,999, generated by using equation B (Dextran molecular weight standards), were the most accurate molecular weight values of these two components.

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