THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

CHROMATOGRAPHIC SEPARATION AND ISOLATION OF SKIN TEST ACTIVE COMPONENTS OF HISTOPLASMIN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Norman, Oklahoma

1967

CHROMATOGRAPHIC SEPARATION AND ISOLATION OF

SKIN TEST ACTIVE COMPONENTS OF HISTOPLASMIN

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

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

Sincere appreciation is recognized for the generous assistance extended by numerous colleagues and technical personnel.

I am grateful to the members of the dissertation committee for critically reading the manuscript.

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CHAPTER I

INTRODUCTION

The medical significance of histoplasmosis has become increasingly evident in the last two decades. Reported cases of this disease in human beings and lower animals have risen sharply since Darling's initial observation in 1905 (6). Loosli, et al. (25) suggested that 30,000,000 people in the United States alone have been exposed to the etiological agent Histoplasma capsulatum, as determined by a positive skin test reaction to histoplasmin. Furcolow (13) estimated that 500,000 new cases of histoplasmosis could be expected each year in the United States.

Numerous epidemiologists have studied the geographic distribution of human histoplasmosis utilizing roentgenography and detection of infection allergy. Christie and Peterson (4) and Palmer (31) pioneered such epidemiological surveys in the early 1940's. Endeavors by these investigators and others (10, 14, 33, 45) have been partially successful in delineating endemic areas of histoplasmosis (Figure 1). The major obstacles in interpretation of these data have been variability of skin test antigens and cross reactivity with other mycoses.

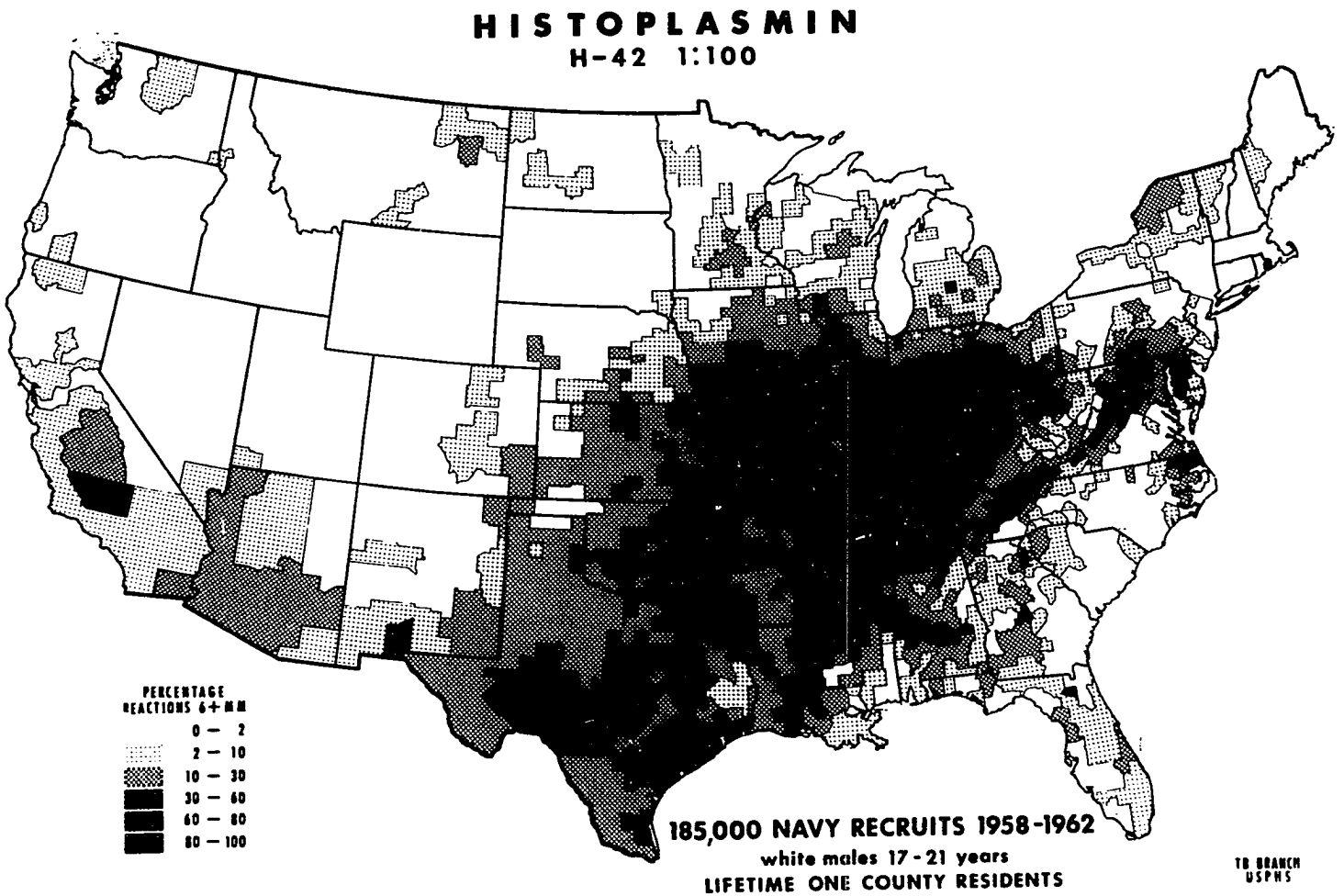


Figure 1. Geographic Distribution of Histoplasmin Skin Test Sensitivity.

The antigens most often used for detection of infection allergy in early epidemiological investigations and used by most clinicians at the present time are crude mycelial growth filtrates of H. capsulatum. Van Pernis, et al. (48) were first to use a crude mycelial growth filtrate for the study of histoplasmosis in 1941. They demonstrated cutaneous reactions in an infected human patient, experimentally infected mice and performed serological tests (complement fixations and qualitative precipitin tests) utilizing a 24 to 48 day old glucose broth filtrate as antigen. Zarafonetis, (51) utilizing William's synthetic medium, prepared a 7 week old mycelial growth filtrate which he called "histoplasmin". Christie (4) produced skin test antigen used in the initial survey conducted by Peterson and himself, using dextrose broth for cultivation of the mycelial phase of H. capsulatum. This is referred to as the "Vanderbilt Product."

Emmons (11) recognized the necessity for standardization of histoplasmin. He used Smith's asparagine medium (41) for the production of histoplasmin H-3 and determined its skin test potency by intradermal inoculation of H. capsulatum sensitized guinea pigs.

By 1945, the United States Public Health Service had become aware of the need for large scale epidemiological studies of histoplasmosis and practicing physicians were insisting on the availability of a reliable skin test product for clinical use. Since a large quantity of standardized H-3 was not available several relatively small lots of histoplasmin were prepared by Howell (40) and later pooled to form the skin test product designated as H-15. It was prepared using Long's synthetic medium and was standardized by comparison with H-3 histoplasmin

using the technique of Emmons (11).

When wide spread use of H-15 was adopted in 1946, it was thought that a large enough quantity had been prepared to suffice for a number of years. However, the supply was so depleted by 1948 that a new product was needed. Accordingly, another relatively small pooled lot, designated H-40, was prepared for interim use until a more adequate supply could be provided. After testing several proposed lots, a new pooled product designated H-42 was selected (40).

H-42 histoplasmin is the product most widely used today and it is estimated that the supply now available will suffice for several years. Major pharmaceutical companies, in recent years have begun to produce and distribute histoplasmin commercially. Eli Lilly Company is currently marketing a product for serological use and detection of infection allergy.

Numerous investigators have attempted to fractionate various yeast cell preparations or filtrates of H. capsulatum. The basic objective, in most instances has been to increase specificity or sensitivity of the antigen or antigens involved in serological and infectious allergic reactions.

Van Pernis, et al., in 1941 precipitated a polysaccharide complex from histoplasmin with acetone (48). This complex elicited a positive skin test reaction in a patient with histoplasmosis. A few years later Salvin and Ribi (26) were able to separate the protoplasm from the intact yeast cell wall of H. capsulatum and demonstrate that both components participate in infectious allergic reactions. However, only the cell wall appeared to contain the antigenic component responsible

for the fixation of complement in the presence of specific antisera.

Canadian investigators (24) have extracted yeast cells of H. capsulatum with a variety of organic solvents (pyridine, urea, acetone, phenol, trichloroacetic acid and etc.) showing that specific components participate in serological reactions. These investigations also suggest that antibodies, elicited by the various chemically characterized components in human and experimental infections, appear at different times during the course of the disease (22 & 23).

Other investigators have prepared active skin test substances by ethanolic precipitation and various deproteinization procedures (5, 8, 20, & 35). More recent approaches have utilized various cellulosic ion exchangers. Greene, et al., (16) exploiting such techniques have been able to isolate the "h" and "m" antigens of Heiner (18) from crude histoplasmin. Markowitz (27) and Fadula (12) also utilizing DEAE-cellulose have been able to corroborate the findings of Greene in isolation of serologically active fractions of histoplasmin. Fadula (12) also has demonstrated similar phenomena for several mycelial filtrates of Histoplasma duboisii.

The development, in recent years of various cross-linked dextran polymers has allowed a new approach to fractionation procedures based on molecular size and thus indirectly molecular weight. Hook, et al., (19) separated crude histoplasmin and yeast cell filtrates on a column of Sephadex G-200. High molecular weight fractions, separable from low molecular weight medium constituents and metabolic products, were serologically active in indirect fluorescent antibody tests, complement fixation tests and precipitin tests.

The investigation reported here involves separation and collection of skin test active components of histoplasmin by such column chromatographic procedures. It would seem reasonable that if the skin test active component of histoplasmin could be separated from other extraneous substances, it could be dehydrated and thus lend itself to quantitation in terms of dry weight of active substance. This would be desirable not only from the practical standpoint of processing, handling, and preservation, but also from the scientific point of view. Partial purification of the skin test active component of histoplasmin would be a step in the direction of elucidation and comprehension of its antigenic complexity.

In the mycobacterial field, the progress toward this goal received its impetus with the preparation of a purified protein derivative (PPD) of tuberculin (39) and, more recently, of other antigens (1, 9). Even though PPD is not a chemically pure substance, the active principle of tuberculin has been identified as a protein or protein complex. The dose of PPD is therefore expressed as milligrams of protein complex per unit volume of diluent.

The objective of the following series of investigations was to separate and isolate the principal skin test active component from crude histoplasmin. It also was anticipated that the dry weight or chemically estimated "skin test dose" could be determined.

CHAPTER II

MATERIALS AND METHODS

Preparation of Histoplasmins. Histoplasmins were prepared by quantitative inoculation of sterile Smith's asparagine medium (41). A 50 liter pyrex carboy containing the sterile medium was inoculated with a mycelial suspension of H. capsulatum. Aliquots (500 ml) of the homogenous inoculated medium were dispensed into 1 liter cotton plugged Erlenmeyer flasks. The flasks were incubated at 27 C and approximately 75% humidity to reduce dehydration with prolonged incubation. The flasks also were overlaid with foil allowing only the cotton plugs to be exposed to a General Electric germicidal lamp, to reduce contamination.

Histoplasmins were harvested after 5 months incubation by introduction of merthiolate (1:10,000) and double filtration through a Seitz filter. Crude histoplasmins were dispensed into 100 ml antigen bottles, sealed, labeled and stored at 4 C until fractionation. Table 1 illustrates the isolates of H. capsulatum and inocula used in preparation of the histoplasmins.

Fractionation of Histoplasmins. Aliquots of histoplasmin H-42 and histoplasmins produced in our laboratory using the Scritchfield and Grand Island isolates of H. capsulatum were fractionated on a 2.5 x 40 cm

Table 1. Preparation of Histoplasmin

H. capsulatum	Culture Age (days)	Histoplasmin	Inoculum Size	
			Total Particles/ml	Viable Particles/ml
Scritchfield	17	HT-1 A-5	1.5×10^3	1.0×10^1
Scritchfield	25	HT-1 B-5	1.0×10^5	2.3×10^3
Grand Island	16	HT-2 A-5	4.5×10^4	4.6×10^1
*H-42		H-42	-	-

*Courtesy of Dr. Carroll E. Palmer, U. S. Public Health Service

column of Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden). The fractionations were continuously monitored at wavelengths of 260 and 280 millimicrons utilizing a Model 2000 multiple sample absorbance recorder. The effluent was collected in 2.5 ml aliquots and consecutive aliquots were pooled according to peaks of 280 mu absorption. The Sephadex G-25 column, Model 2000 multiple sample absorbance recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and fraction collector are shown in Figure 2. All fractionations were performed at 20 C.

The pooled aliquots were concentrated under vacuum at 4 C. Table 2 indicates the original volume of crude histoplasmin introduced onto the Sephadex G-25 column and the volume to which each respective fraction (pooled aliquots) was restored for skin testing and chemical characterization.

The fractions in turn were tested for detection of "infection allergy" by intradermal inoculation of infected and normal guinea pigs. Inoculum size was 0.1 ml and all induration readings reported were taken 48 hours after initial inoculation.

Chemical Characterization of Histoplasmins. Protein was determined quantitatively by the modified Folin-Phenol method of Lowry (26), using crystalline bovine serum albumin as standard. Absorption at 280 mu also is indicative of proteinacious material (15 & 49). Various histoplasmin fractions reacted with triketohydrindene hydrate (ninhydrin) which is a sensitive reagent for amino acids and amino acid residues (17).

Carbohydrate was determined with the anthrone reagent (28 & 38) and by the phenol-sulfuric acid technique of Dubois (7), using D-glucose as standard. Reducing sugars were determined colorimetrically using the

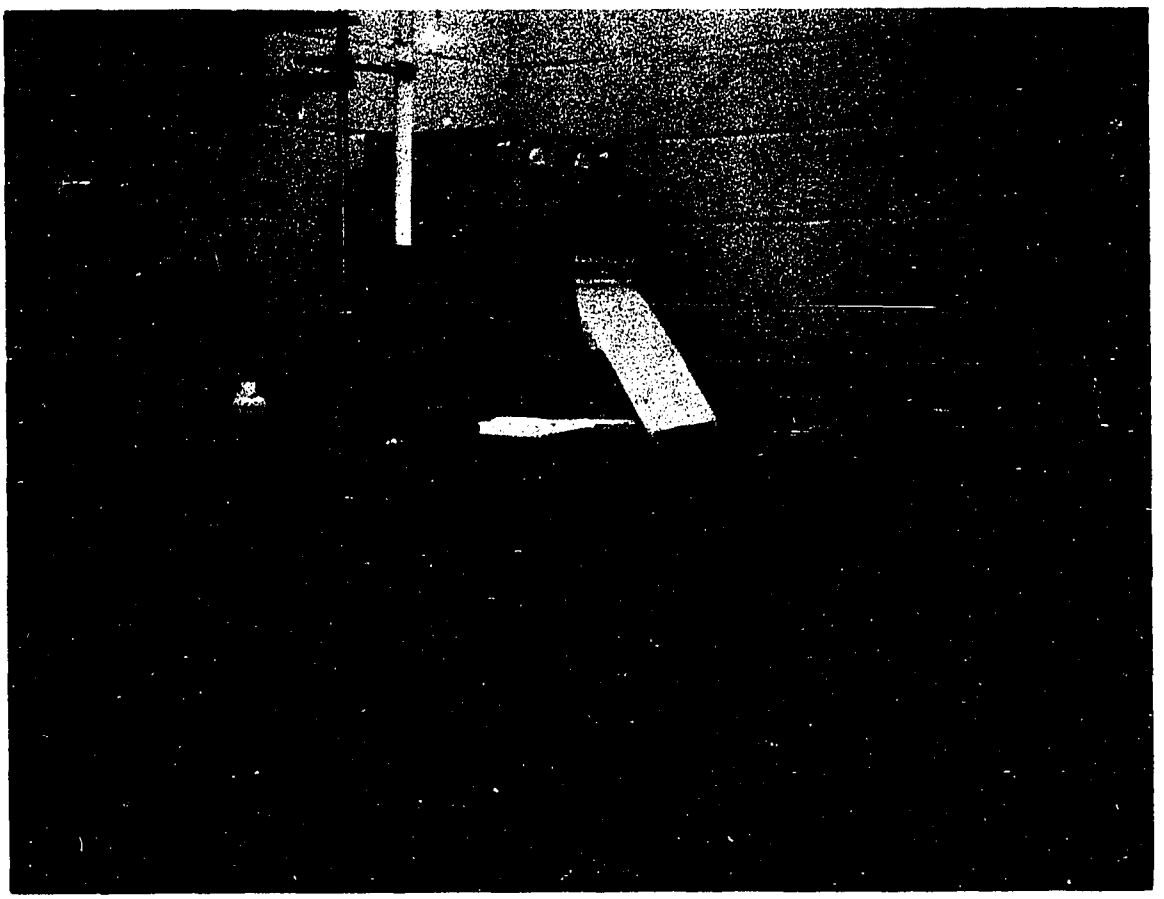


Figure 2. Apparatuses for Chromatographic Fractionation of Histoplasmin.

Table 2. Fractionation of Histoplasmin

Histoplasmin	Fractionation No.	Sample Size Volume (ml)	Fractions Volume (ml) after concentration
HT-1 A-5	*1.	25	5
	2.	30	10
HT-1 B-5	*1.	20	10
HT-2 A-5	*1.	20	10
H-42	*1.	5	5
	*2.	25	25

*Specific histoplasmin and its respective fractions tested for skin test potency and chemically characterized.

arsenomolybdate and copper reagents of Somogyi (43). Qualitatively, the presence of nucleoprotein was based on the principle of Warburg and Christian (49), and confirmed by a positive diphenylamine reaction (29).

Electrophoretic Characterization of Histoplasmins. Crude histoplasmins and their respective fractions were subjected to paper electrophoresis at various hydrogen ion concentrations for 12 to 24 hr at 200 v. Phosphate and veronal buffers of pH 5.5, 7.0 and 8.6 were prepared using (8.7 g KH_2PO_4, 0.38 g Na_2HPO_4), (3.5 g KH_2PO_4, 5.7 g Na_2HPO_4) and (2.8 g Barbital, 20.6 g Na-Barbital) per liter of distilled water respectively. Electrophoretic studies were performed using an E-C Pressure-plate Electrophoresis Cell (E-C Apparatus Corp., Philadelphia, Pa.) and Whatman No. 3 mm paper strips. Paper strips were developed with ninhydrin (17) and alkaline silver nitrate (47).

Skin Testing with Histoplasmins. Male albino guinea pigs approximately 6 months of age were used throughout these studies. All animals were tested initially with histoplasmin H-42 (1:25) and those exhibiting a positive reaction, i.e. 5mm or greater induration at 48 hr, were eliminated from the study.

Skin test negative animals (300-1000 g) were arranged by weight according to a preselected scheme into 51 regiments of 4 animals each. Sensitization was incurred by intraperitoneal inoculation of a viable mycelial suspension of H. capsulatum (Scritchfield) in a total volume of 1 ml. Viability was determined by quantitative inoculation of mycelial suspension onto the surface of Sabouraud's dextrose agar plates. Plates were incubated at 25 C and colonies counted after an incubation period of 15 to 20 days. Table 3 shows the isolates of H. capsulatum, inoculation schedule and dose used to induce sensitization.

Table 3. Sensitization of Guinea Pigs

Test Group	*H. capsulatum* Isolate	Culture Age (days)	Inoculation Schedule (day)	Inoculum Size	
				Total Parts	Viable Parts
HT-1	Scritchfield	30	1	9.4×10^6	1.4×10^4
	Scritchfield	34	5	1.4×10^7	3.7×10^5
	Scritchfield	41	11	2.2×10^7	5.5×10^5
	Scritchfield	14	42	6.8×10^6	6.5×10^4
	Scritchfield	28	46	1.7×10^7	1.1×10^4
	Scritchfield	22	50	1.6×10^7	1.0×10^5
	Scritchfield	41	119	1.4×10^5	3.4×10^6
	Scritchfield	61	185	1.6×10^5	2.2×10^4

Control (non-infected) animals were randomly distributed throughout the regiments. The number varied from 10% initially to 25% as the number of infected animals decreased with terminal disease and death.

Crude histoplasmins and their respective fractions were assayed for detection of "infection allergy" by intradermal inoculation (Mantoux test) of various concentrations of protein and carbohydrate or dry weight of material dissolved in 0.1 ml of diluent. Erythema and induration were measured and recorded 24 and 48 hr after inoculation. However, only 48 hr induration readings were used for the statistical calculations and thus for interpretation of results.

CHAPTER III

RESULTS

Crude histoplasmins were subjected to fractionation on all grades of Sephadex (G-200, G-150, G-100, G-75, G-50, G-25, G-15, and G-10) currently marketed by Pharmacia Fine Chemicals. It was determined that maximal separation of high molecular weight compounds passing the column early in the elution scheme was obtained with G-25.

Histoplasmin H-42. Figure 3 illustrates the elution pattern for a 25 ml sample of histoplasmin H-42 from the Sephadex column. The arrows indicate the pooled 2.5 ml aliquots and thus represent the fractions. Respective fractions from left to right or with increasing tube number were labeled according to lower case Greek letters, Alpha, Beta, Gamma, Delta, Epsilon, Zeta, Nu, and Theta. The broken line represents the 280 mu readings expressed in per cent transmission along the ordinate axis and the dotted line the corresponding 260 mu readings.

Absorption at 280 mu indicates a small quantity of proteinacious material in the second or Beta fraction and a considerable quantity in the Zeta fraction. Also absorption at 260 mu, according to the principles of Warburg and Christian (49) would imply the presence of nucleoprotein in Delta, Epsilon and Zeta fractions with peak concentration in Delta.

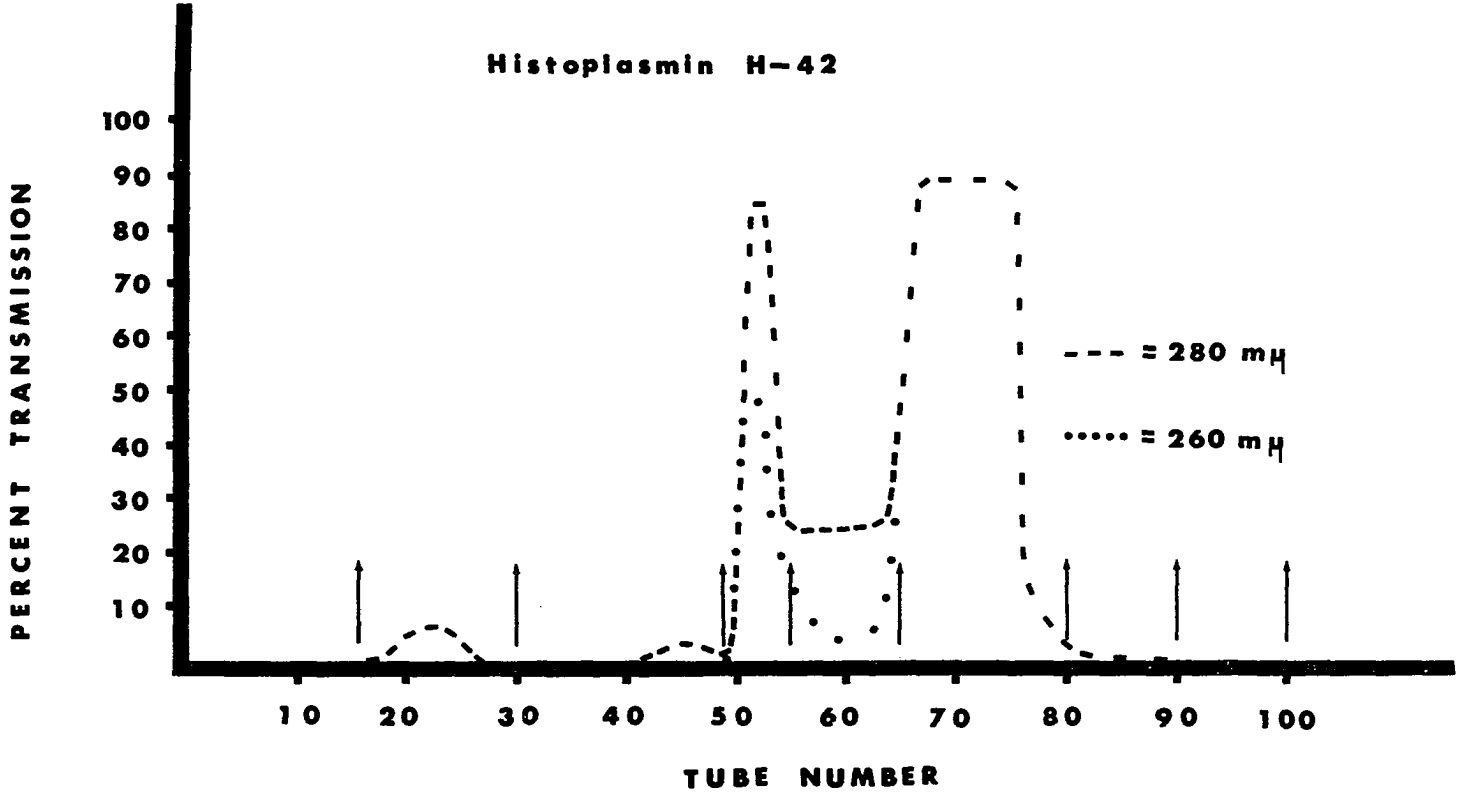


Figure 3. Elution Pattern for Histoplasmin H-42 from Sephadex G-25 Column measured at 260 mu and 280 mu.

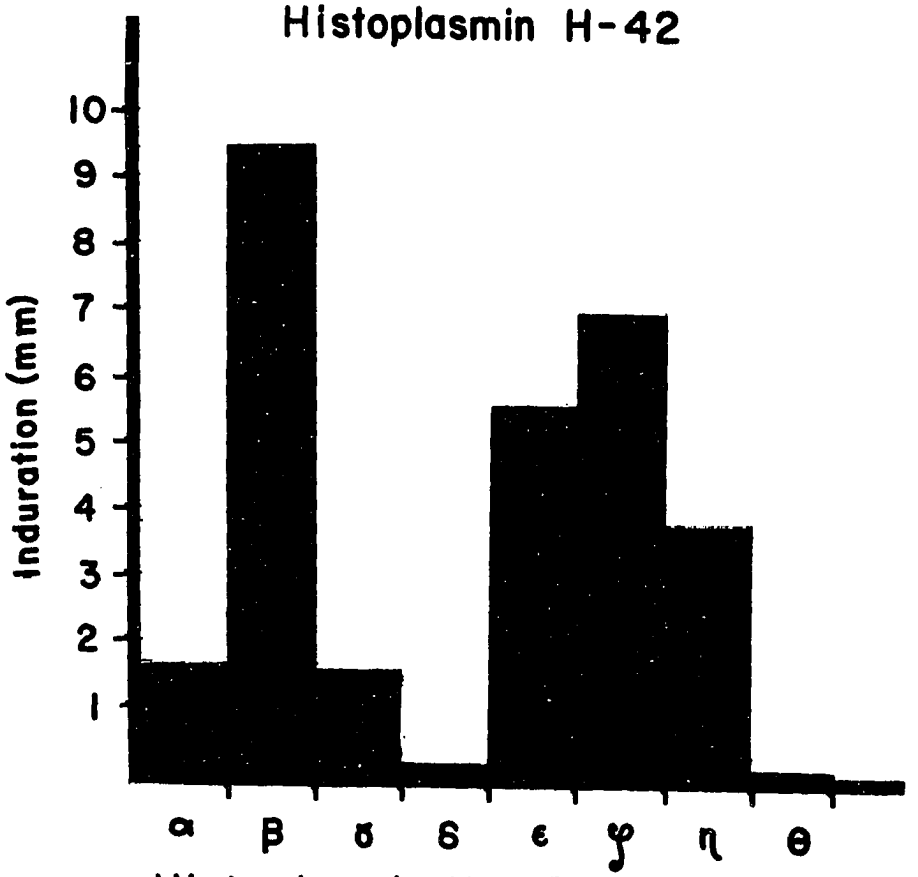
It also is significant that 260 mu absorption indicates the absence of nucleoprotein in the second or Beta fraction. These observations from spectrophotometric data were confirmed by the diphenylamine test (29).

One observes from Table 2 that the H-42 fractions were concentrated to initial starting volume of crude histoplasmin H-42 fractionated. This theoretically should restore any individual component or components occurring in a single fraction to its original concentration. Thus the fraction containing the active component or components should elicit a comparable cutaneous reaction to crude histoplasmin H-42 (1:25) upon testing infected and control animals. The only other alternative is the active component is present in more than one fraction, in which case adjacent fractions should exhibit similar but varying degrees of smaller positive reactions.

The table at the bottom of Figure 4 shows the colorimetric determination of sugar and protein expressed in micrograms per ml for the respective histoplasmin fractions. Total quantity of protein or carbohydrate per skin test dose can be determined by moving the decimal one place to the left, since volume of inoculum was 0.1 ml. The bottom line represents the ratio of carbohydrate to protein.

The histogram at the top of Figure 4 illustrates the mean results of animal testing. The ordinate axis shows 48 hr. induration expressed in millimeters and the abscissa the respective H-42 fraction tested.

These data indicate two active fractions: Beta exhibiting maximal skin test activity and containing very little protein and carbohydrate and Zeta having considerable protein but negligible



Carbohydrate	µg/ml							
Anthrone	0	8	52	0	2	0	58	0
Phenol-H_2SO_4	0	8.6	46	3.2	8	2	60	0
Protein	µg/ml							
Lowry	0	8.7	13.4	425	250	5400	115	0
Ratio (C/P)	-	.95	3.7	.004	.02	.0002	.51	-

Figure 4. Chemical Characterization and Results of Skin Testing Histoplasmin H-42 Fractions.

carbohydrate. It also is noteworthy that the ratio of carbohydrate to protein is very near one in the Beta fraction.

Electrophoresis studies of crude histoplasmin H-42 (1:25) shows 3 bands sensitive to ninhydrin (Figure 5). All 3 bands occurred in the Beta fraction but only one was stained with alkaline silver nitrate.

Histoplasmin HT-1 A-5. Figure 6 shows the elution pattern for a 25 ml sample of Scritchfield or HT-1 A-5 histoplasmin. The pattern is similar to that obtained for H-42 except the integrated area under the 280 mu absorption curve implies considerably less total protein. This fact also is supported by the Lowry (26) protein determinations. The arrows again represent the respective pooled 2.5 ml aliquots or fractions labeled by lower case Greek letters.

The chemical characterization and results of animal testing for the HT-1 A-5 fractions are illustrated in Figure 7. Beta fraction again yields maximal skin test activity and possesses only a small amount of protein and carbohydrate. The Nu fraction has the largest quantity of protein, 2200 ug/ml or 220 ug/test and shows the second peak of animal activity.

A 25 ml sample originally was applied to the Sephadex G-25 column and the various fractions concentrated to 5 ml (Table 2). This represents a 5 fold concentration of the various HT-1 A-5 fractions with respect to original sample. Figure 7 shows 18 ug/ml of protein occurring in the HT-1 A-5 Beta fraction. This is twice the concentration of protein occurring in H-42 Beta fraction (Figure 4). It also is observed that a slightly larger, though not statistically significant, cutaneous reaction resulted (Figure 4 & 7).

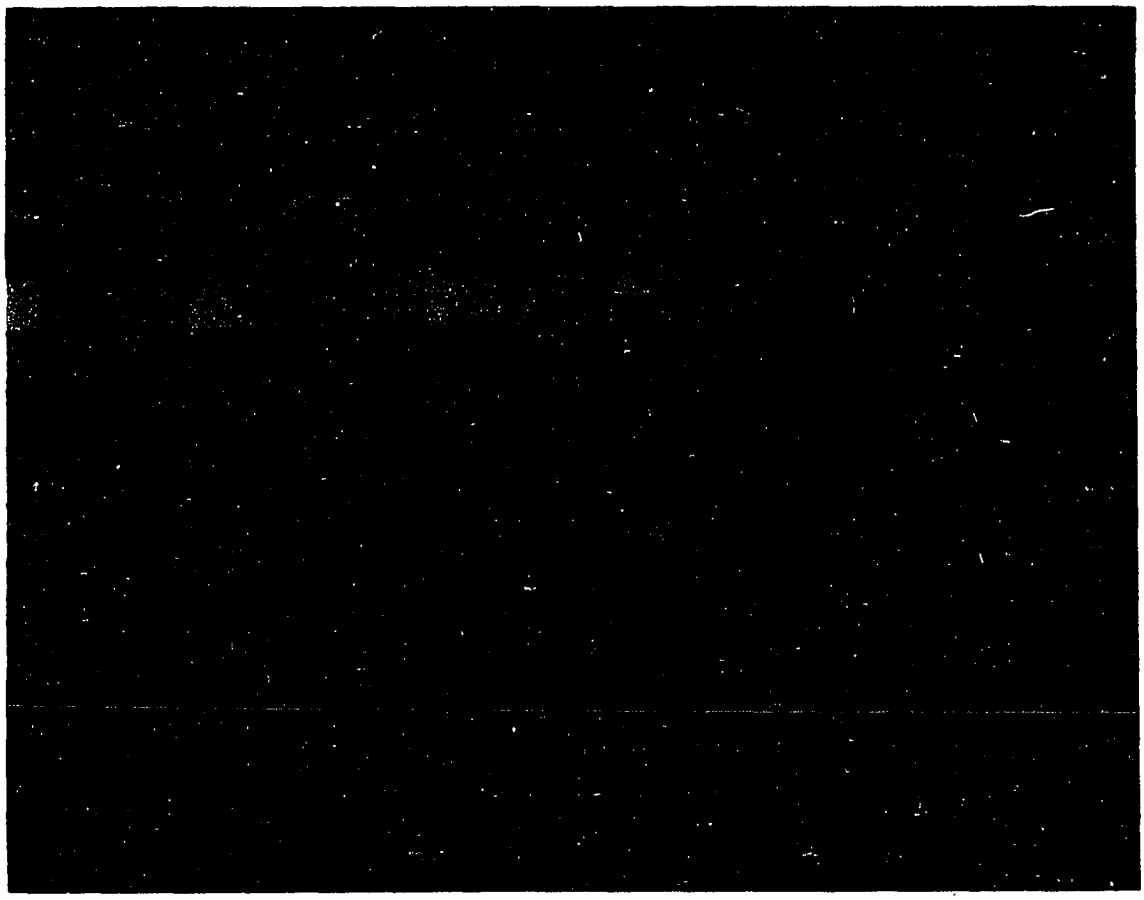


Figure 5. Electrophoresis Paper Strips of Histoplasmin H-42, Crude Histoplasmin HT-1 A-5 and Histoplasmin HT-1 A-5 Fractions Stained by Ninhydrin and Alkaline Silver Nitrate Methods.

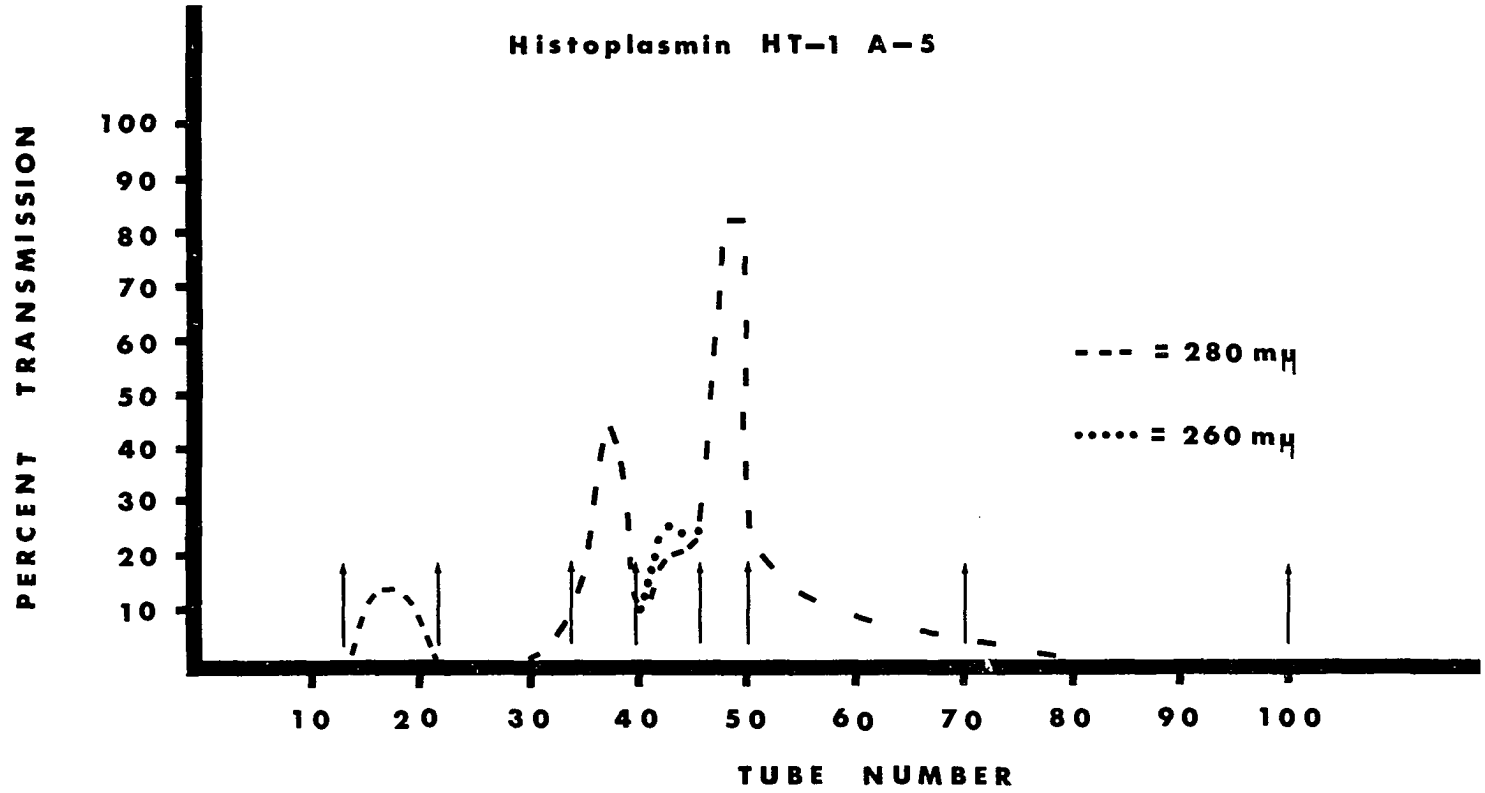
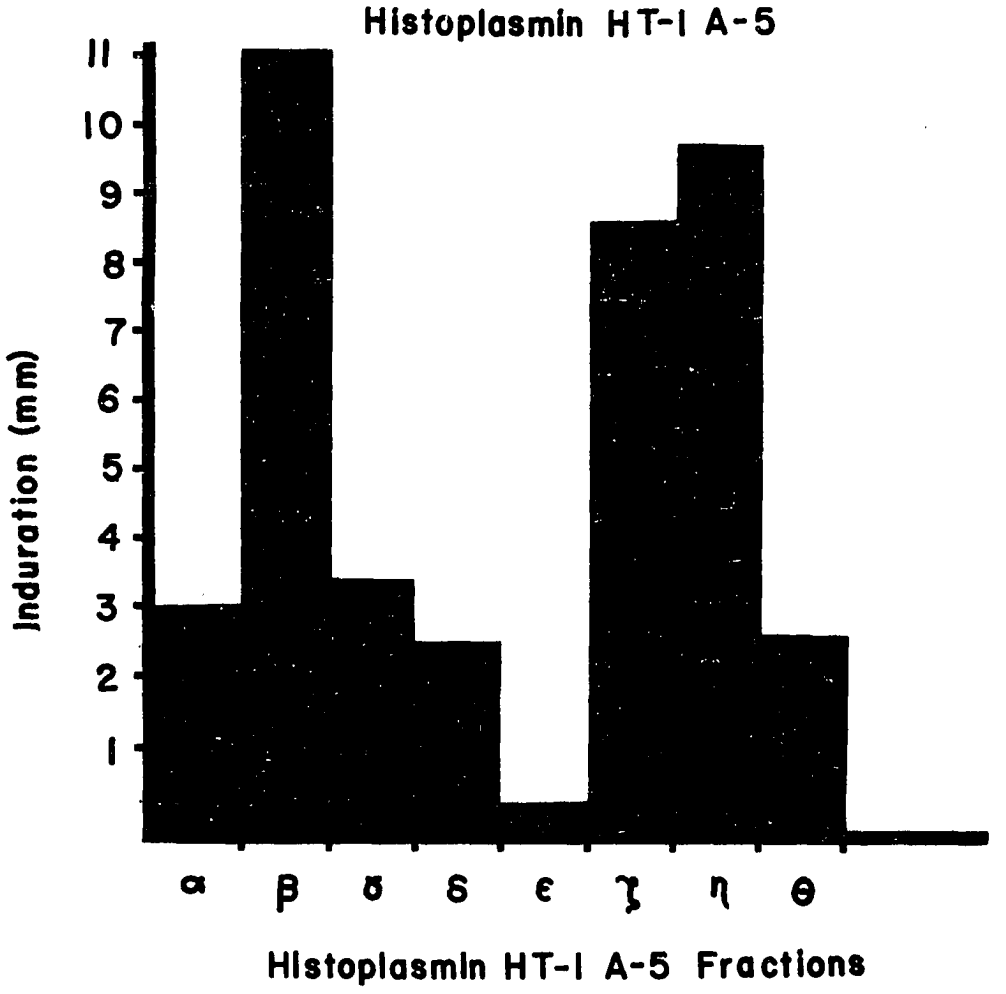


Figure 6. Elution Pattern for Histoplasmin HT-1 A-5 from Sephadex G-25 Column measured at 260 mu and 280 mu



Carbohydrate	**µg/ml**							
Anthrone	0	12.5	2800	205	2100	2750	1260	82
Phenol-H_2SO_4	0	8	1610	275	5650	5500	1800	300
Protein	**µg/ml**							
Lowry	0	18	210	197	450	1250	2200	115
Ratio (C/P)	-	0.6	10.5	1.2	8.6	3.3	0.7	1.7

Figure 7. Chemical Characterization and Results of Skin Testing Histoplasmin HT-1 A-5 Fractions.

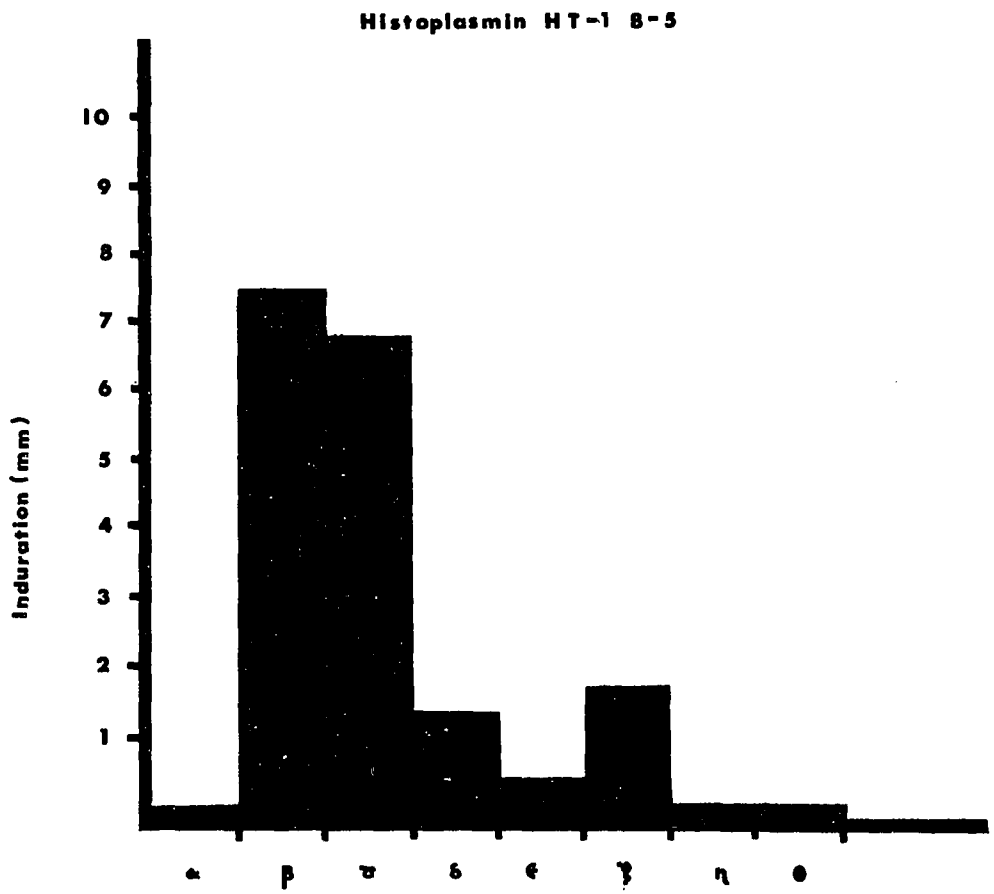
Electrophoresis studies of crude HT-1 A-5 histoplasmin showed 4 ninhydrin sensitive bands (Figure 5). An additional band (blue-black band of strips 59 and 60) probably occurred but was masked due to high protein concentration. Strip 60 shows 3 bands for HT-1 A-5 Beta fraction. The purple band nearest the origin occurs in approximately the same position as the carbohydrate (47) band of strip 168.

Similar electrophoresis of Smith's asparagine medium and solutions of its nitrogen containing components demonstrated the second band to the right of the original in crude HT-1 A-5 histoplasmin (strip 90) was asparagine and the fourth glycine (blue). Asparagine also was confirmed by the staining method of Pasieka (32). The characteristic staining reaction and mobility of glycine also accounts for 1 of the 3 bands occurring in crude H-42 histoplasmin and its respective Beta fraction.

Histoplasmin HT-1 B-5. Histoplasmin HT-1 B-5 also was prepared using the Scritchfield isolate of H. capsulatum. However, the initial inoculum was 200 times greater than for HT-1 A-5, with respect to viable particles/ml (Table 1) and the mycelial mat became submerged in the liquid medium between the second and third month of growth.

The Beta fraction (Figure 8) contained 1.33 ug of protein per test and elicited a mean skin test reaction slightly less than H-42 Beta fraction (0.87 ug protein/test) and HT-1 A-5 Beta fraction (1.88 ug protein/test). However, the HT-1 B-5 fractions were tested after the H-42 and HT-1 A-5 fractions, at which time the sensitized guinea pigs appeared to have waned with respect to reaction to crude H-42 (1:25).

Figure 9 shows that the fractionation for HT-1 B-5 was not as



Carbohydrate	**µg/ml**							
Anthrone	0	11	18	100	3050	6250	3500	0
Phenol-H_2SO_4	0	14.5	40.5	100	2200	4650	2400	0
Protein	**µg/ml**							
Lowry	0	13.3	71.7	221.7	820	710	0	0
Ratio (C/P)	-	0.96	0.41	0.45	3.2	7.68	-	-

Figure 8. Chemical Characterization and Results of Skin Testing Histoplasmin HT-1 B-5 Fractions.

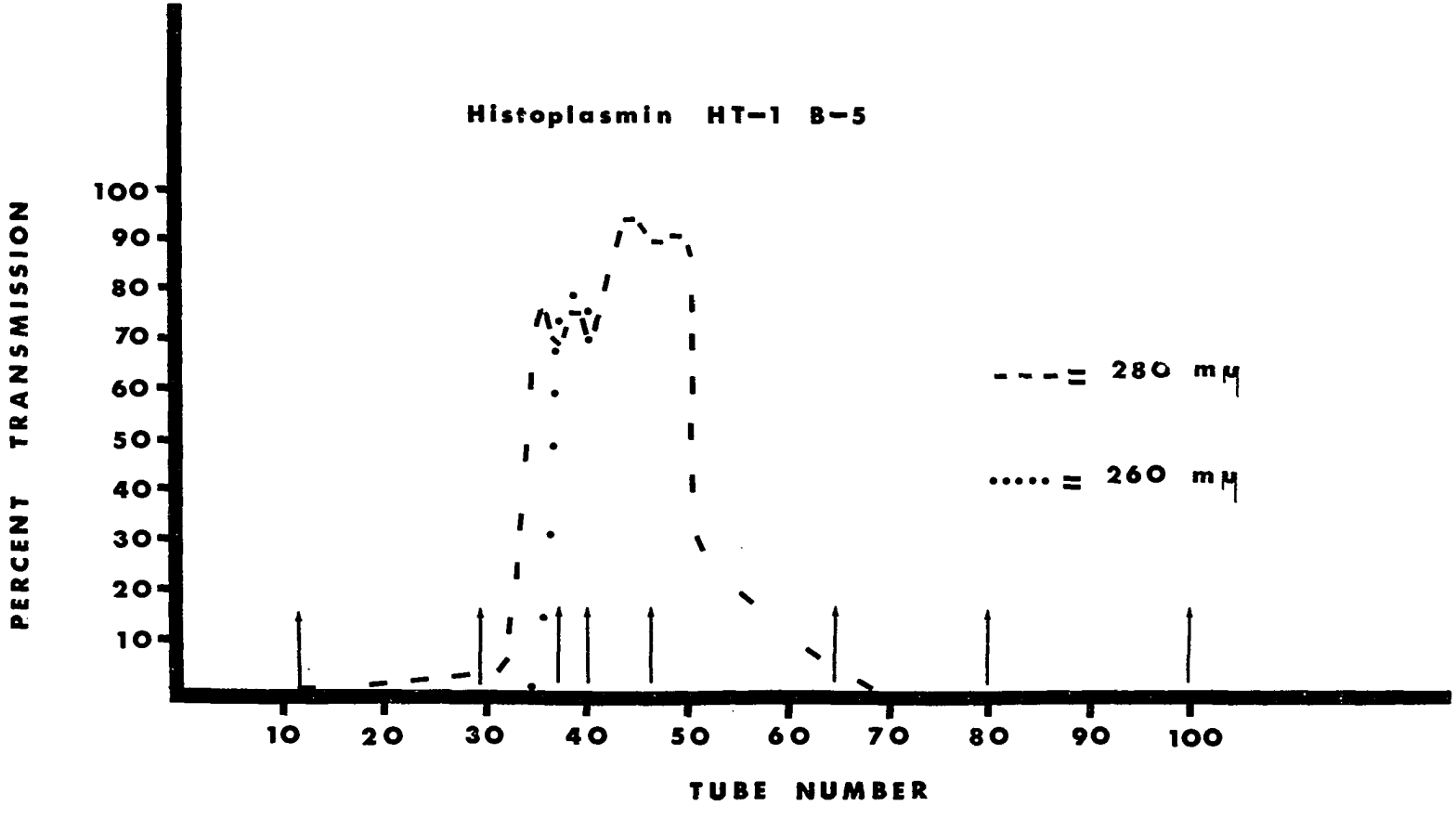


Figure 9. Elution Pattern for Histoplasmin HT-1 B-5 from Sephadex G-25 Column measured at 260 mu and 280 mu.

discrete as for histoplasmins H-42 and HT-1 A-5. This is reflected in a greater skin test response to HT-1 B-5 Gamma fraction (Figure 8).

The secondary peak of skin test activity characteristic of H-42 Zeta and HT-1 A-5 Nu was considerably reduced for HT-1 B-5 Zeta fraction. Figure 8 shows a marked reduction in detectable protein possibly explaining the smaller reaction.

Histoplasmin HT-2 A-5. HT-1 A-5 and B-5 involved skin testing studies of animals sensitized with the homologous isolate of H. Capsulatum. It is known from unpublished data (personal communication, Dr. Howard W. Larsh) that the Scritchfield isolate produces a potent crude skin test antigen. Therefore, heterologous or HT-2 A-5 histoplasmin and its respective fractions were tested on Scritchfield sensitized guinea pigs.

Figure 10 shows the indiscrete elution pattern for a 20 ml sample of HT-2 A-5 histoplasmin. Figure 11 illustrates the results of skin testing Scritchfield sensitized animals. The Beta and Gamma fractions elicited cutaneous reactions comparable to H-42, HT-1 A-5 and HT-1 B-5 Beta fractions.

Various concentrations of Histoplasmins. The results presented above indicate two distinct fractions of the various histoplasmins examined are eluted from the Sephadex G-25 column which elicit skin test activity. Figure 12 shows the results of skin testing HT-1 (Scritchfield sensitized) animals with similar protein concentrations of the various histoplasmin fractions. Beta and Gamma fractions were either concentrated or diluted to 1.5 ug/test and the remaining fractions diluted to 10 ug/test. Alpha and Theta fractions were not altered since they normally contained little or no protein and elicited insignificant skin reactions.

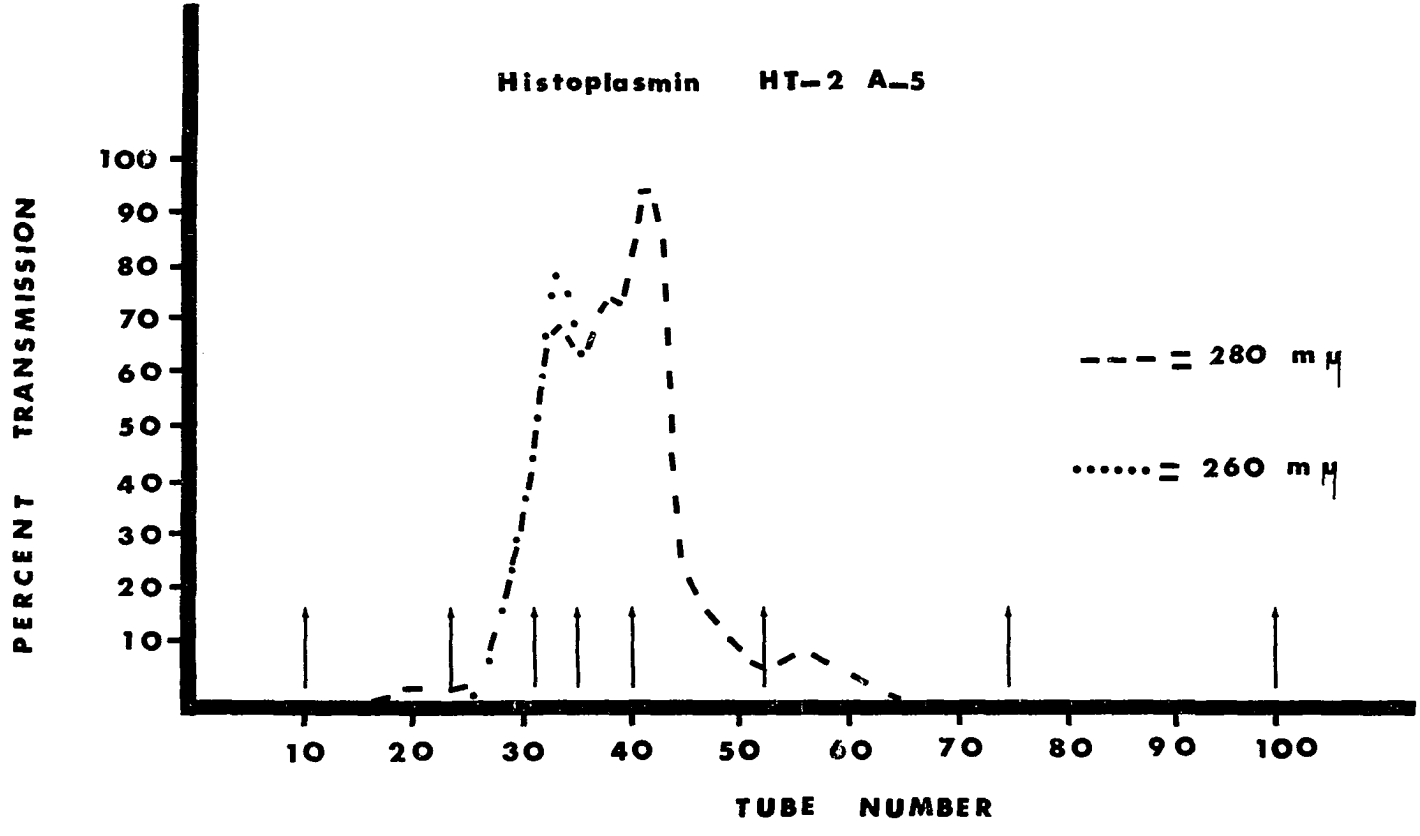
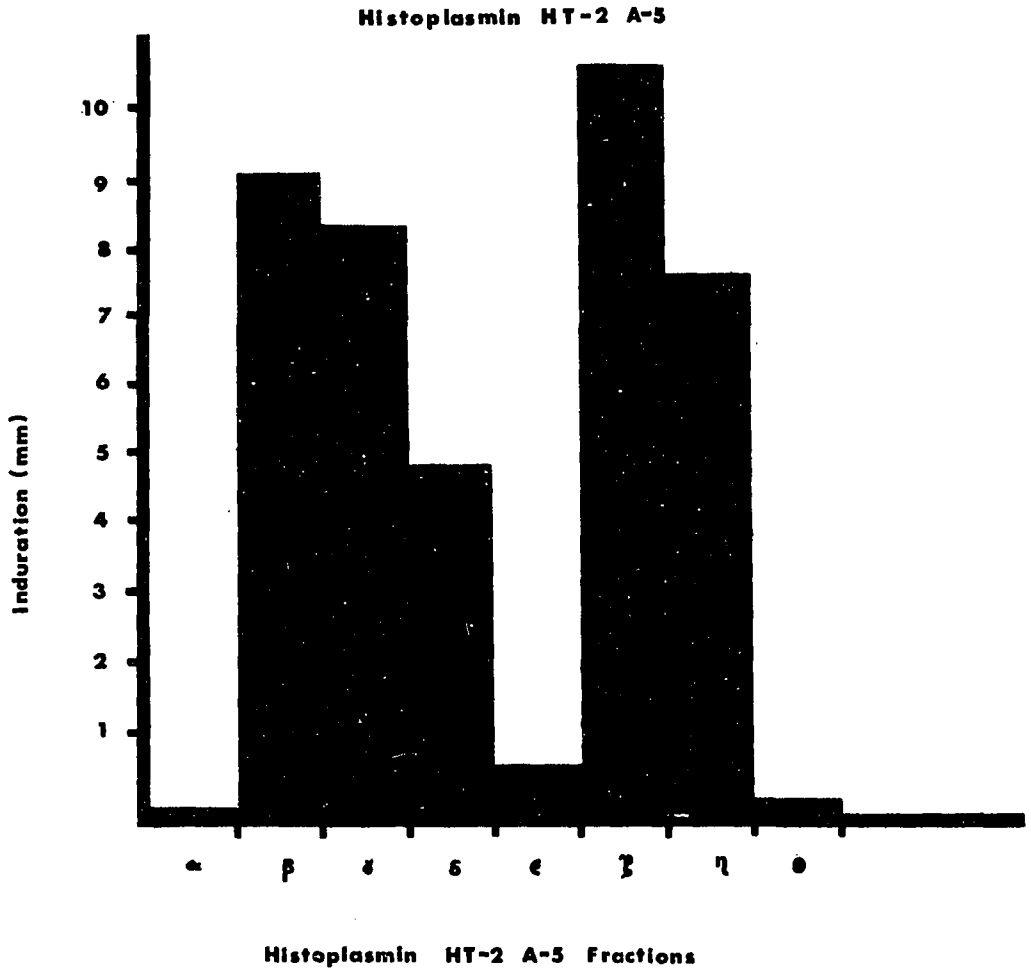
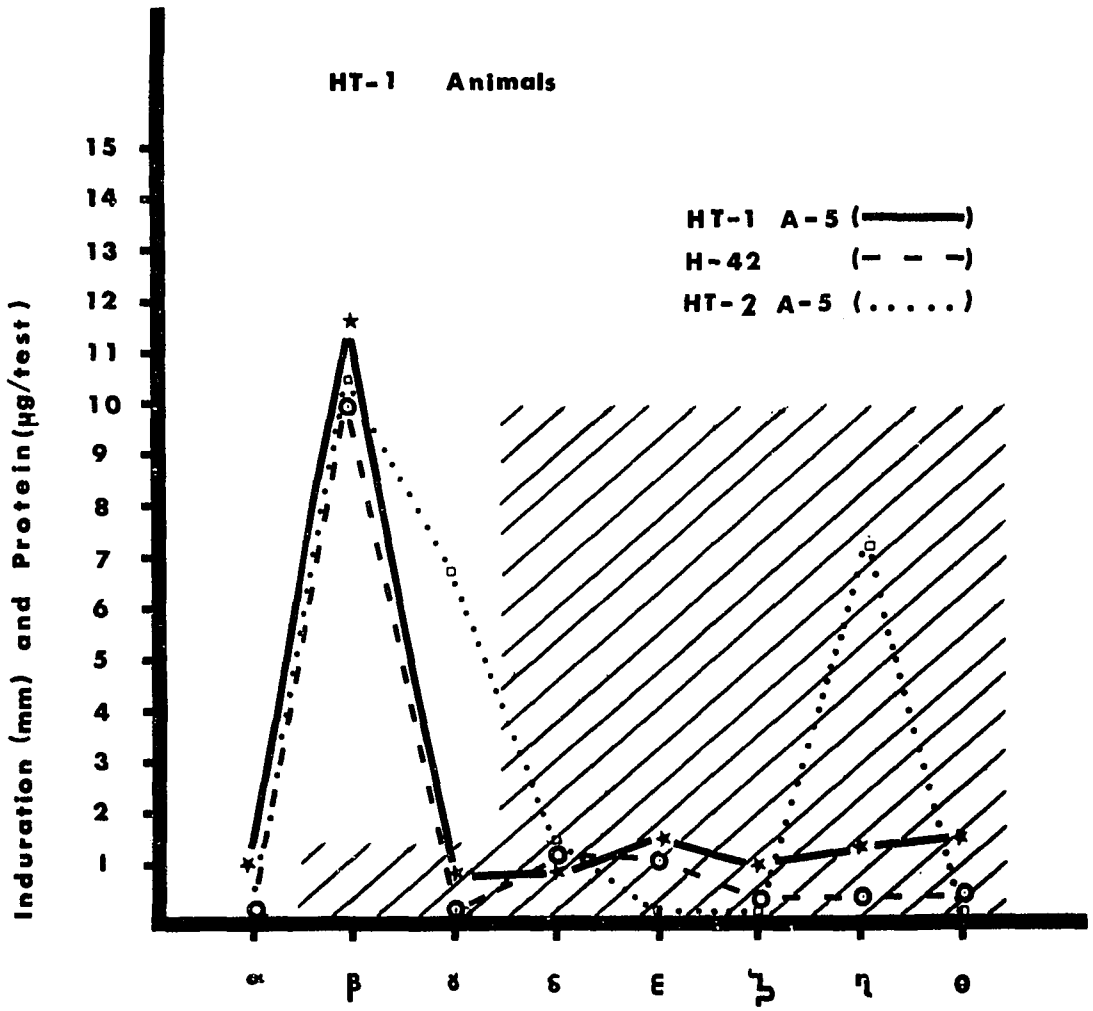


Figure 10. Elution Pattern for Histoplasmin HT-2 A-5 from Sephadex G-25 Column measured at 260 mu and 280 mu.



Carbohydrate µg/ml								
Anthrone	0	52	19	2200	10^3	1100	50	0
Phenol-H_2SO_4	0	57	58	2400	5850	500	70	0
Protein µg/ml								
Lowry	0	6.6	26.6	363	430	500	26.6	0
Ratio (C/P)	-	8.3	1.5	6.3	18	1.6	2.3	-

Figure 11. Chemical Characterization and Results of Skin Testing Histoplasmin HT-2 A-5 Fractions.



	Carbohydrate (µg/test)							
HT-1 A-5	0	1.03	16.5	122	86.7	73.8	7	5.5
H-42	0	1.42	4.9	.75	2	.08	51.3	0
HT-2 A-5	0	1.0	3.8	18.54	1843	33	13	0

Figure 12. Chemical Characterization and Results of Skin Testing Various Protein Concentrations of Histoplasmins H-42, HT-1 A-5 and HT-2 A-5 Fractions.

These data (Figure 12) indicate that H-42 and HT-1 A-5 fractions of high protein concentration i.e. Epsilon, Zeta, and Nu essentially lose their activity at lower concentrations. However, HT-2 A-5 Nu fraction remained active. The concentration of this fraction was not increased to 10 ug/test but retained at 2.66 ug/test. This represents its protein concentration at initial testing (Figure 11) and its mean reaction size of 7.4 mm induration is almost identical to that obtained at initial testing (7.6 mm). Beta fractions for all histoplasmins remained active, in most instances eliciting skin test reactions comparable to those obtained at initial testing (Figure 4, 7, and 11).

Histoplasmin Purified Derivative. HPD was prepared by lyophilization of two separate 10 ml volumes of concentrated H-42 Beta fraction. The dry material was weighed and then re-dissolved in sterile non-pyrogenic distilled water. The stock solutions were diluted to desired concentrations using volumetric pipettes.

Figure 13 shows the results of animal testing with various concentrations of re-dissolved HPD. Therefore it appears that 60 ug or 0.06 mg of HPD yields a 10 mm cutaneous reaction.

Protein and Total Carbohydrate in Histoplasmin. Table 4 shows protein and total carbohydrate detected in the various crude histoplasmins. Histoplasmin H-42 (1:25) appears to contain approximately 2 1/2 times more protein than other histoplasmins.

pH of Histoplasmin. Figure 14 shows the pH of various histoplasmins used in these investigations. Mean values were obtained using Beckman Zeromatic and Fisher model 210 pH meters. A gradual decrease was observed with increasing age in most instances. A sharp increase to pH 6.3 was observed for histoplasmin HT-1 B-4.

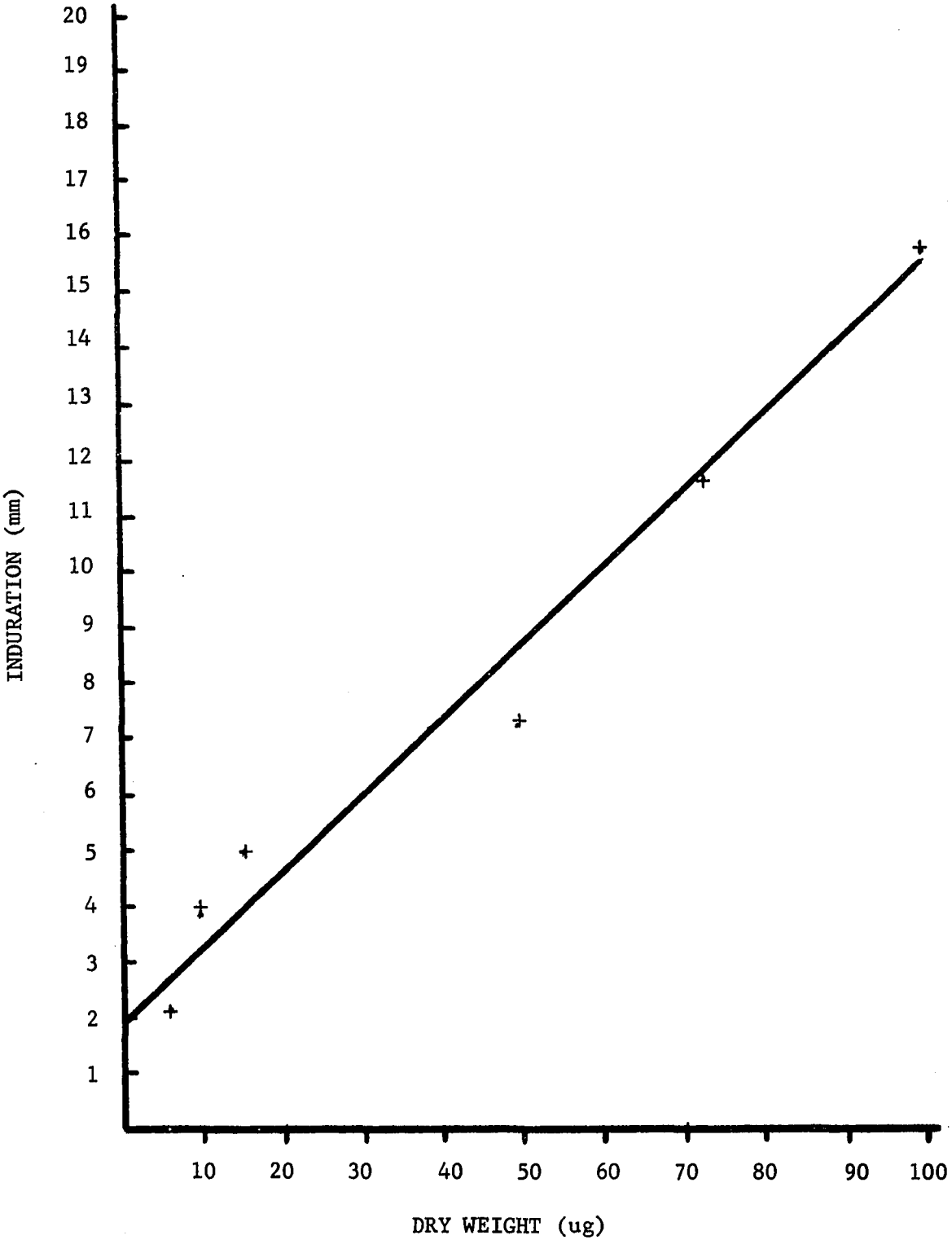


Figure 13. Dry Weight vs. Induration for HPD.

Table 4. Protein and Total Carbohydrate Concentrations of Crude Histoplasmins.

Histoplasmin	Protein ug/ml	Carbohydrate ug/ml
HT-1 A-5	1377	12,000
HT-1 B-5	1520	12,000
HT-2 A-5	1355	9,200
H-42(1:25)	38,500 *	*

*Phenol interferes with Anthrone and Phenol-H_2SO_4 determinations, and Lowry Protein determination.

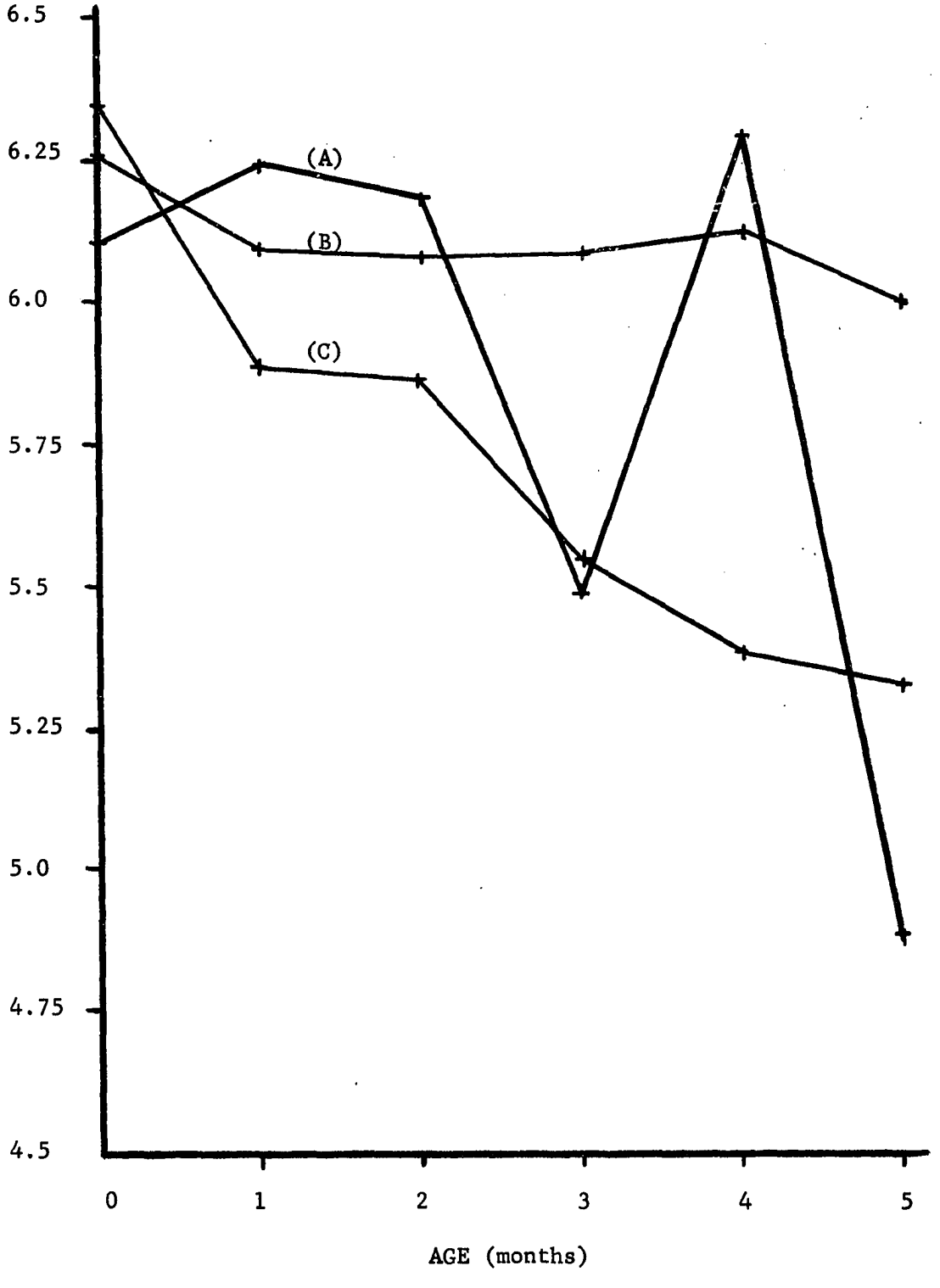


Figure 14. Variation of pH with Increasing Age of Histoplasmin.
A = Histoplasmin HT-1 B-0 to HT-1 B-5.
B = Histoplasmin HT-2 A-0 to HT-2 A-5.
C - Histoplasmin HT-1 A-0 to HT-1 A-5.

CHAPTER IV

DISCUSSION

Disease prognosis and epidemiological investigations have provided the impetus for research of fungal antigens and their role in infection allergy. Evidence presented in this paper shows that the major component of crude histoplasmin active in detection of infection allergy can be separated and isolated from extraneous low molecular weight medium constituents and metabolic products. The significance of this achievement has already been indicated.

Previous investigations along these lines have dealt primarily with improvement of serodiagnostic procedures. However, for most systemic mycoses serodiagnostic techniques offer little or no insight with respect to disease prognosis. The only immunological tool of any significant value for prognosis appears to involve the mechanism of delayed hypersensitivity. Intradermal inoculation of various fungal antigens apparently reveals an antibody in most persons infected with homologous pathogenic fungi which closely parallels the ability of the patient to resist the disease. This antibody usually is considered to be tissue fixed, although it may exhibit limited circulatory ability due to its genesis. On the other hand, inability to demonstrate its circulatory existence in the hemopoietic or reticulo-endothelial system

may result from lack of a suitable detection technique or our inability to interpret presently existing data. Electrophoretic investigations of convalescent sera reveal several globular proteins which have not been characterized and apparently are associated with various disease processes (42 & 46).

It is known that in endemic areas a high percentage of the population is exposed and infected with the etiological agent of histoplasmosis. Yet, only a very small percentage disseminate and progress to terminal disease. In the majority of cases, acquired immunological resistance develops and spontaneous cure results. This acquired immunological state apparently is of long duration and wanes only in the terminal years of life. Therefore, again the mechanism of delayed hypersensitivity lends itself to important epidemiology of public health.

Most researchers have approached the study of antigens and antigenicity via the yeast phase of this unique diphasic fungus. Salvin, et al., (35) considers that this approach is justified since the yeast-phase cells are manifest in the disease state. Also previous reports of protection against infection involved vaccines derived from the cell wall of the yeast phase of H. capsulatum (21 & 35). However, regardless of authenticity, most intracutaneous testing continues to utilize the mycelial growth filtrate, histoplasmin.

Previous knowledge indicates that the potency of crude histoplasmin is dependent upon the isolate of H. capsulatum used in its production. Hook, et al., (19) have suggested that antigenic specificity and potency might be improved through utilization of genetically homogeneous cultures of carefully selected isolates of fungi. The Scritchfield

isolate used in this study was selected for production of histoplasmins HT-1 A-5 and HT-1 B-5 (Table 1) because previous data (unpublished) indicated its superior antigenicity.

Considerable variation exists in supporting media, physical growth conditions and harvesting age for production of histoplasmin. Media containing organic nitrogenous materials such as peptone or neopeptone are not desirable since they elicit varying degrees of cutaneous reactions. These reactions are easily differentiated by their immediate and nonspecific character but tend to mask the desired delayed immunological response. Histoplasmins used in these investigations were produced in Smith's liquid asparagine medium (41) which contains the amino acid asparagine as the only organic source of nitrogen.

Salvin and Hottle (37) reported that maximal yield of histoplasmin in a glucose asparagine medium was obtained after 2 to 2 1/2 months of growth at room temperature. The appearance of histoplasmin in the medium was paralleled by a rise in alkalinity to pH 8-8.5, a drop in reducing sugars t zero and a decrease in total nitrogen. In contrast Figure 14 sl we a decrease in pH with increased age of histoplasmin to 5 months. Data (Table 5) indicates concentration of total carbohydrate in crude histoplasmins remained essentially the same as initial concentration of glucose occurring in the liquid medium employed in production of histoplasmins (12 g/liter). However, there is a gradual reduction of detectable reducing sugars with increasing age of histoplasmin. This suggests conversion of glucose to non-reducing carbohydrate such as the structural component chitin and possibly skin test active materials.

Several investigators have reported isolation of skin test active components from crude histoplasmin. However, in most cases, the separation and isolation procedures were time consuming and complex involving extremely harsh treatment of the biological product which was obtained in small yield. It was decided that if a suitable separation could be obtained by column chromatographic procedures several of these problems could be eliminated.

The principle of Sephadex chromatography is based on separation of chemical compounds differing in molecular size and thus indirectly molecular weight. Elution may be obtained with physiological saline or distilled water thus eliminating drastic pH changes or high salt concentrations required of ion exchange resins. The relatively mild treatment should increase yield especially if a labile protein or protein complex were involved. The simplicity of eluting fluid should eliminate the necessity for desalting by dialysis or other means.

One major advantage is that one obtains little dilution of the desired compound or component and in fact should get a concentration effect with Sephadex chromatography. This is amply illustrated by fractionation of histoplasmin H-42 (1:25) where a 25 ml sample was applied to the column (Table 2) and Beta fraction collected in 10 (tube 17-27, Figure 3) 2.5 ml aliquots. Figure 4 shows that Beta fraction elicited a mean reaction of approximately 9.5 mm upon animal testing. This is very near the value of 11.? obtained for crude histoplasmin H-42 (1:25).

Table 2 also shows that considerable concentration of HT-1 A-5, HT-1 B-5 and HT-2 A-5 Beta fractions was required to elicit comparable skin test reactions. This re-emphasizes the potency of histoplasmin

H-42 (1:25) and indicates the concentration of active component occurring in other histoplasmins. Comprehension of this point explains the tremendous variation in skin test potency of crude histoplasmins and difficulty encountered in standardization according to presently accepted procedures (30 & 40).

Figures 4, 7, and 11 show a high incidence of reactions on test animals with fractions passing the Sephadex G-25 column late in the elution pattern. The characteristic brown color of histoplasmin was found to be associated with this peak of concentrated protein material. The large skin test reactions were most likely due to the large quantity of protein inoculated: 540 ug/test for H-42 Zeta, 220 ug/test for HT-1 A-5 Nu and only 50 ug/test for HT-2 A-5 Zeta fraction. In contrast to Beta fractions these antigens elicited large reactions on control animals.

It also was observed that the resulting skin test reactions were not similar to those exhibited by the Beta fractions or crude histoplasmin H-42 (1:25). These reactions usually exhibited an immediate erythema or flare reaction which increased in intensity and persisted for 5 to 10 days, on occasion progressing to the definition of an Arthus phenomenon. In contrast, Beta fractions and crude histoplasmin H-42 (1:25) elicited reactions with initial appearance of induration and erythema at 6 to 8 hours. Reaction size characteristically reached a maximum between 24 to 48 hours and usually disappeared by 96 hours.

Rich (34) proposed differentiation of the two types by the slow development of the delayed type reaction following re-exposure to antigen, contrasted to the prompt appearance of the reaction in the Arthus-sensitized body. The above reactions were differentiated by this definition.

Observation of elution patterns and chemical data in figures 4, 7, 8, and 11 indicates H-42 Zeta fraction contains considerably more protein than fractions showing the second peak of animal activity in other histoplasmins. Table 4 shows similar results for total protein in crude histoplasmin H-42 (1:25) as compared to other histoplasmins. These data are ambiguous, since crude histoplasmin H-42 was a 1 to 25 dilution and its respective fractions were restored to initial concentration. Other histoplasmin fractions were 2 to 5 fold concentrations of initial sample volume, thus they should contain considerably more protein. Subsequent findings have shown that crude histoplasmin H-42 (1:25) contains 0.5 to 1 per cent phenol as preservative. Phenol interferes with the Lowry protein test and passes the Sephadex column late in the elution pattern. This undoubtedly explains the indicated high protein concentration in crude histoplasmin H-42 (1:25) and H-42 Zeta fraction. Protein absorption at 280 mu is based on the frequency of occurrence of cyclic amino acids in the protein chain, thus the cyclic structure of phenol passing the Sephadex column in conjunction with H-42 Zeta fraction apparently enhances the absorption at 280 mu.

Analytical gel exclusive chromatography (2 & 3) suggests the skin test active component is of relatively low molecular weight. Fractionation range from Sephadex G-25 is 100 to 5000 molecular weight. This would indicate a molecular weight of 5000 or less for Beta fraction material. If this conclusion is valid, then the active component as a protein would consist of only 50 to 100 amino acid residues. However, no further attempts were made to determine molecular weight and extreme caution should be exercised in estimation of molecular weight from

exclusion chromatography data for crude preparations.

Electrophoresis studies, in all cases showed 3 bands sensitive to ninhydrin and one sensitive to alkaline silver nitrate (47) for the Beta fractions. Alkaline silver nitrate, a general method for detection of sugar (28 & 38), suggests that the Beta fractions do not consist entirely of proteinacious material. Recycling also failed to eliminate the alkaline silver nitrate sensitive bands from the Beta fractions. It must be remembered that Sephadex gel is a dextran and that autogenous hydrolysis could allow slight contamination of eluate with sucrose or its glucose and fructose components. However, this phenomenon should occur only at low frequency in which case the contaminating sugar would appear in close proximity with exogenous glucose substrate in late low molecular weight fractions.

Comparison of colorimetrically estimated protein to dry weight of H-42 Beta fraction (HPD) also indicates that the skin test active component consists of material associated with protein (Table 4). These data indicate approximately 10% of total materials was detected as protein. Dayson and Evans (8) have demonstrated that deproteinization of skin test active antigens destroys skin test reactivity. Investigators (44 & 50) have reported similar inactivation of antigens used in serodiagnostic procedures. Salvin (35) reported isolation of a skin test active antigen from crude histoplasmin which appeared to be a complex containing carbohydrate and protein moieties. His electrophoretic data also suggested a protein fraction nearest the anode containing little carbohydrate was active in producing skin test reactions. Salvin in addition suggested that occurrence of multiple protein components in

electrophoresis strips of active fractions probably was due to "gradual release of the active protein from immobile carbohydrate."

Dayson et al. (8) reported cutaneous reactions of 5 to 7 mm induration on H. capsulatum infected rabbits with 10 ug of antigen. The antigenic fraction was obtained by ethanolic precipitation of yeast cell filtrates. Other investigators (5, 20 & 35) have reported skin test reactions of approximately 10 mm induration elicited with 10 to 100 ug of antigen obtained by similar chemical precipitation of yeast cells or filtrates. Figure 12 shows that approximately 60 ug of HPD elicited skin test reactions of 10 mm induration. This is comparable to that elicited by crude histoplasmin H-42 (1:25).

Although other investigations have reported antigens apparently active in detection of infection allergy the procedures are expensive and extremely laborious. Also only small yields of active material, insufficient for large scale distribution were obtained from large volumes of yeast cell filtrates. Past experience has shown that a very small percentage of crude histoplasmin lots possess sufficient potency to warrant standardization and subsequent distribution for skin test purposes. Evidence presented in this paper indicates that yields of active material may be obtained rapidly and with relative ease from crude histoplasmins. Thus crude histoplasmin lots possessing mediocre skin test potency could be utilized without complete loss.

Evidence also indicates that skin test material may be dehydrated to a solid state which is easily re-dissolved in physiological saline or non-pyrogenic distilled water. Existence of solid antigens reduces the laborious task of processing and standardization, reduces chances of contamination and enhances preservation.

CHAPTER V

SUMMARY AND CONCLUSIONS

Numerous attempts have been made to fractionate yeast cell preparations or filtrates of H. capsulatum. Most investigators have approached this problem through ethanolic precipitation or the use of various cellulosic ion exchangers. These procedures are expensive, laborious and yield only small quantities of skin test active material exhibiting considerable variability.

Evidence presented in this paper indicates crude histoplasmins may be fractionated by Sephadex G-25 chromatography, resulting in quantitative yield of skin test active material. Data obtained from fractionations of four separate histoplasmin lots indicates the procedure is applicable in varying degrees of efficiency to histoplasmins of varying degrees of skin test potency.

It also appears that spectrophotometric absorption at a wavelength of 280 millimicrons is directly proportional to quantity of skin test active material occurring in various crude histoplasmins.

Colorimetric estimation of protein, carbohydrate, and electrophoretic data suggest that the skin test active component may be a protein-carbohydrate complex. Other data indicate the skin test active complex is either grossly contaminated with inert materials of

approximately similar molecular weight or consists of a major component complexed with a protein moiety.

Attempts in past years to dehydrate crude histoplasmin to a dry state have been unsuccessful. Such endeavors have resulted in a gummy colored mass which could not be restored to a liquid phase. However, it has been possible to dehydrate the Beta fraction of crude histoplasmin to a powdered state. The powder is easily re-dissolved in sterile non-pyrogenic distilled water or physiological saline and apparently retains its skin test activity.

The principal contributions of these investigations have been: 1. Isolation of the skin test active component from crude histoplasmins, 2. Development of rapid and relatively simple methods applicable to large scale fractionation and isolation of the skin test active component from crude histoplasmin, 3. Suggestion that the skin test active principle is in fact a protein-carbohydrate complex, and 4. Indications that dehydrated skin test active material may be quantitated in terms of dose weight.

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